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GREEN TECHNOLOGIES FOR INNOVATIVE MATERIALS PRODUCTION FOR ACTIVE FOOD PACKAGING

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“It always seems impossible, until it's done”

[Nelson Mandela]

GREEN TECHNOLOGIES FOR INNOVATIVE MATERIALS PRODUCTION FOR ACTIVE FOOD PACKAGING

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AIM AND CONTENTS OF THE Ph.D. THESIS

The main purpose of the work was to investigate the behavior of different biopolymers enriched with natural compounds processed with different mild technologies for the production of flexible films with the aim of creating innovative bio-based materials with a potential application in the field of active food packaging. The main challenges faced in this work are attributed to the choice of materials and processes used, guided by the desire to find alternative materials to polymers of fossil origin and synthetic additives, with the additional ability to counteract food degradation by preserving shelf-life, in order to go against the growing trend of food waste. Starting from the choice of materials, the investigated polymers were mainly two, zein, a protein extracted from corn, and polycaprolactone, a synthetic polyester, both chosen for their biodegradable nature and for their suitability as food contact materials recognized by the Food and Drug Administration. The second challenge of this work was represented by the choice of manufacturing processes for the polymeric films. In fact, different technologies have been investigated both conventional, such as solvent casting, and innovative, such as electrospinning, and the impregnation process with supercritical fluids, with the aim of producing different polymer structures with consequent different properties.
The topic addressed by this thesis is organized and subdivided into chapters as follows.

Chapter 1- State of the Art - the content of this introductory chapter derives from extensive literature review of the food packaging field. After a brief reference to traditional materials, the innovations of the food packaging sector represented by the concepts of active and intelligent packaging were illustrated in detail.

Chapter 2- Development of zein-based polymeric films for active food packaging - in this chapter, different production techniques of zein-based flexible films, and different loading techniques of natural compounds have been compared. In particular, zein films were functionalized with vanillin as an antimicrobial agent and with a natural extract rich in antioxidant molecules obtained from spent coffee grounds.

Chapter 3- Development of polymeric blend by solvent casting - this section was dedicated to improving the mechanical properties of zein films through the production of a polymer blend based on zein and chitosan.

Chapter 4- Development of polycaprolactone films for active food packaging - this chapter was focused on the production of polycaprolactone films functionalized by the loading of fatty acids as potential phase change materials through the electrospinning process. In addition, the supercritical

fluids impregnation process was investigated for the loading of antioxidant agent, alpha-tocopherol, on polycaprolactone supports produced by electrospinning and solvent casting.

Chapter 5- Electrospinning economic and financial analysis - a business plan was carried out to evaluate the profitability of antimicrobial zein films produced by electrospinning to be commercialized as antimicrobial patches to be inserted inside the primary packaging for fresh food with high-value density.

Chapter 6- General Conclusions - a final discussion was conducted based on the results obtained during the thesis work identifying both the strengths and the limits of the processes and of the products obtained.

ABSTRACT

The Food and Agriculture Organization (FAO) reports that 1.6 billion tons of food are wasted every year in the world, which corresponds to about 1/3 of all food production destined for human consumption. Food waste weighs on a global level as it brings with it a series of problems related to resources such as water, land, energy, labor, capital, the mismanagement of which entails an economic burden of about 680 billion dollars in industrialized countries. The food products most subjected to waste are the fresh ones, with a shelf-life of a few days and the products for which the cold chain is required, such as refrigerated and frozen foods. However, for the latter products, compliance with the cold chain along the entire distribution line cannot unfortunately be guaranteed. These impacts derive both from the unreliable management of transport and marketing, and from the limited thermal insulation capacity of traditional materials such as plastic and cardboard. Furthermore, in parallel with food waste, the second global problem is the increase and accumulation of waste deriving from disposable food packaging of fossil origin. In fact, with increasing industrialization, food and packaging have become a single system whereby if one is wasted, the other is wasted as well. In fact, just think that from the 1950s to 2015, plastics production reached 8.3 billion tons, throwing away about 6.3 billion in nature, of which 79% ended up in landfills and in natural environments, 12% was incinerated and only 9% was recycled.

In recent years the situation has improved, in fact, in 2020, according to Eurostat data, 23% of plastic waste produced in Europe ended up in landfills, 42% was incinerated and 35% was recycled.

However, these data also show that the objectives set by the European Union, namely the achievement of 50% plastics recycling by 2025 and 55% by 2030 are still far. This demonstrates how the use of biodegradable polymers or biopolymers is increasingly becoming a need rather than a choice. Hence the need to find solutions to minimize if not cancel the effects of these two problems. A potential solution is offered precisely by packaging, conceived not in the traditional sense of the term, i.e., as systems for the containment and generic protection of food from the external environment, but in an innovative way for which packaging has become the protagonist and not a passive part of the food product. With this in mind, the so-called active packaging is designed with the aim of interacting in a controlled way with food in order to preserve its nutritional properties, organoleptic properties and extend its shelf-life, all in a safe way for the consumer and for the manufacturer. To perform this function, active packaging is designed through the incorporation of components capable of releasing or absorbing substances from packaged foods or from the environment surrounding the product and, moreover, systems defined as intelligent packaging allow to monitor the quality of products in real time. Among active packaging, antimicrobial and antioxidant films appear to be the most promising to achieve these goals. While, there is little literature on packaging capable of preserving food from sudden changes in temperature, but a

possible approach to control and maintain a desired temperature, for a limited period of time, seems to be represented by the thermal energy storage approach.

Therefore, this Ph.D. work was aimed at developing films based on natural polymers, such as the protein zein and the polysaccharide chitosan, and synthetic but biodegradable polymers such as polycaprolactone to investigate the potential of these materials to be used as active packaging. The polymeric matrices obtained were then functionalized by adding natural active ingredients such as vanillin, present in vanilla pods, characterized by antimicrobial activity, spent coffee grounds extract, rich in caffeine and polyphenols, alpha-tocopherol, contained in olive oil with high antioxidant properties and finally, natural phase change materials such as fatty acids to study the feasibility of developing packaging materials with thermal insulation properties. For film production, traditional methods, such as extrusion and molding, are mainly based on the direct loading strategy. However, these techniques have some drawbacks related to the use of toxic and polluting solvents, high temperatures, low penetration of the active agent into the polymeric substrate and reduced loading efficiencies. For this reason, several greener techniques have been investigated, more suitable for the treatment of natural substances, such as electrospinning, solvent casting and spin coating. Only solvents accepted by the Food and Drug Administration were selected for treatment. Furthermore, the use of an indirect loading technique, the impregnation with supercritical fluids, for the loading of the active agents subsequent to the production of the polymeric supports was also studied. The obtained products were characterized mainly in terms of morphology, migration tests in different food simulants, gas barrier properties, mechanical tests and functional activities through the comparison of the materials and techniques used. The results obtained made it possible to identify the strengths and limitations of both the materials and the techniques used. However, it was possible to identify a potential intended use for all the materials optimized and identify possible improvement methods to upgrade these materials.
Finally, the economic feasibility of the antimicrobial constructs produced by electrospinning through the production of a business plan was assessed.

SUMMARY

1 STATE OF THE ART 1

1.1 Evolution of food packaging concept in history 1

1.2 Classification and Functions of packaging 2

1.3 Food Packaging Materials 3

1.3.1 Paper and Cardboard 4

1.3.2 Metals 5

1.3.3 Glass 6

1.3.4 The plastics dilemma 7

1.3.5 Biodegradable polymers and biopolymers 11

1.3.6 Innovations in food packaging field 24

1.3.7 Active packaging and shelf-life 25

1.3.8 Active packaging and cold chain 30

1.3.9 Intelligent packaging 34

1.4 Regulatory Aspects 41

1.5 Methods for manufacturing polymeric films 42

1.5.1 Extrusion Methods 42

1.5.2 Molding Methods 43

1.5.3 Calendering 44

1.5.4 Solvent Casting 44

1.5.5 Spinning Methods 45

1.6 Requirements and properties of active food packaging 49

2 Development of Zein-based polymeric films for active food packaging 53

2.1. Production and morphological optimization of zein films by electrospinning and solvent casting techniques 53

2.1.1 Materials and methods 54

2.1.2 Results and discussion 56

2.2 Vanillin loading as antimicrobial agent: techniques comparison 64

2.2.1 Materials and Methods 65

2.2.2 Results and discussion 69

2.3 Loading of spent coffee grounds extract on different zein supports 91

2.3.1 Materials and Methods 91

2.3.2 Results and discussion 97

3 DEVELOPMENT OF POLYMERIC BLEND BY SOLVENT CASTING 111

3.1. Materials and Methods 111

3.1.1 Polymeric blend formulation 111

3.1.2 Morphological characterization 112

3.1.3 Mechanical properties 112

3.1.4 Wettability 112

3.2 Results and Discussion 113

3.2.1 Polymeric blend optimization 113

3.2.2 Morphological characterization 114

3.2.3 Mechanical properties 115

3.2.4 Wettability 118

4 DEVELOPMENT OF POLYCAPROLACTONE FILMS FOR ACTIVE PACAKGING 120

4.1. Development and morphological optimization of electrospun PCL processed using green solvents 121

4.1.1 Materials and Methods 121

4.1.2 Results and discussion 122

4.2 Loading of fatty acids as potential phase change materials in electrospun PCL 126

4.2.1 Materials and Methods 126

4.2.2 Results and Discussion 128

4.3 Loading of alpha-tocopherol in different optimized PCL supports by supercritical fluids impregnation 141

4.3.1 Materials and Methods 141

4.3.2 Results and Discussion 143

5 ELECTROSPINNING ECONOMIC AND FINANCIAL ANALYSIS 159

5.1 Economic analysis 159

5.2 B2B Model 160

5.3 Financial Analysis 161

6 GENERAL CONCLUSIONS 170

References 173

1 STATE OF THE ART

1.1 Evolution of food packaging concept in history

The development of food packaging has always been linked to the development of civilization. The need to find ways to contain and preserve food marks the transition from a nomadic lifestyle, in which man procured food to consume it immediately, to the settlement of the first stable communities and the development of the first agricultural practices. Leaves, wood, shells, animal organs and everything that nature could offer was used to contain food. The first ceramic and glass containers date back to 7000 B.C., and the processing industrialization of these materials is attributed to the Egyptians in 1500 B.C. [1]. It was then with the Industrial Revolution that the surplus of production made the conservation and transport of food crucial and led to the development of new processes and materials for packaging. Among the innovations that have marked the history of food packaging, symbolically represented in Figure 1, it must be remembered the hermetic closure of the glass jars by the Frenchman Nicolas Appert in 1810, adopted during the Napoleonic campaigns [2]; the microbiology studies conducted by Louis Pasteur, on the fermentation of beer and the use of heat as a storage medium, which led to the discovery of the pasteurization process in 1862 [3]. In the twentieth century, plastic and metal materials were discovered and established in food packaging, starting with the fortuitous discovery of cellophane, literally "diaphanous cellulose", by the Swiss Jacques Edwin Brandenberger in 1908 [4], without forgetting the fundamental role of aluminum cans during World War II.

Although the plastics industry dates back to 1860, it is then in the first half of the 1900s that these materials take over with the birth of the first supermarkets and the self-service sales method, for which labels also began to be printed with a consequent increase the importance of packaging aesthetics [5]. In this period, packaging became an important marketing tool.

It is clear how food packaging, which today is almost unnoticed by consumers if not for color and claims rather than for functionality, has always been subject to a slow and inevitable process of innovation, sometimes also thanks to ingenious mistakes, due to changing habits and historical periods and as such, it is still evolving today. In fact, from the end of the 1980s an important concept was introduced in the world of packaging, that of smart packages, defined by Wagner as “doing more than just offer protection.

They interact with the product, and in some cases, actually respond to changes” [6]. Even today, this way of conceiving packages represents the innovation of our century for the packaging field.

Figure 1: Timeline of historical contributions to packaging development.

However, since there can be no innovation without tradition, it is necessary to start from the latter and define the concept of packaging as well as the basic functions and traditional materials, before entering the heart of this work, the innovation in food packaging.

1.2 Classification and Functions of packaging

In 1997, Lockart defined packaging as a "socio-scientific discipline which operates in society to ensure delivery of goods to the ultimate consumer of those goods in the best condition intended for their use" [7]. In 2003, Coles et al., argued that packaging should aim at maximizing product sales and minimizing distribution costs [8]. According to Robertson, in 2013, "without packaging, material handling would be a messy, inefficient and expensive exercise and modern consumer marketing would be virtually impossible" [6]. In all these definitions, reference is made to the generic term “packaging”, which includes different types of products, some better known by consumers, others less known, and which must be distinguished.

Generic packaging can be classified into primary, secondary or tertiary, based on its positioning with respect to the product. In particular, primary packaging is placed in direct contact with the product, such as cans, bottles and plastic bags, designed to be sold as a single unit together with the product. A secondary or multiple packaging constitutes a grouping of primary packaging that does not come into contact with the contained products. Tertiary packaging, also called transport packaging, is the set of primary and secondary packaging, used to facilitate transport and logistics, such as cartons containing other multiple packs or wraps around pallets and that often does not even come into

contact with the final consumer. A second classification of packaging can be made on the basis of the mechanical characteristics of the material used, so they can be divided into: flexible, semi-flexible or rigid. Flexible packaging has a shape influenced by the product it contains, such as paper sheets, aluminum or plastic films. Semi-flexible packaging, on the other hand, has intermediate properties between flexible and rigid packaging, and are, for example, cardboard boxes. Finally, rigid packaging is non-deformable and includes wooden crates, glass bottles and metal cans [9].

It is interesting the different approach that consumers have towards packaging, with which they come into contact only when it has reached the end of its life. Some people base the choice of products to buy exclusively based on the most attractive packaging, while other consumers consider it superfluous and harmful to the environment [6]. A common factor is that most consumers often ignore the different functions that a package performs.

The main functions attributed to food packaging are traditionally four:

- **Containment:** it is the oldest function, far from trivial for some products, such as liquids or powdery/granular products that have greater containment needs at any stage of their production, storage and transport cycle.
- **Protection:** a packaging preserves quality and safety, preventing unwanted physical and chemical changes due to light, oxygen and humidity and protects food from external contamination caused by dust, bacteria and parasites, or from leaks, acting as a passive barrier with the external environment [10].
- **Communication:** the packaging plays an important marketing role through labeling, it can communicate information on the foods contained, the nutritional values, and provide instructions for the correct use and storage of the product. According to an old saying “a package must protect what it sells and sell what it protects” [11].
- **Convenience:** as previously mentioned, the production and sale of ready-to-use foods increased significantly at the end of the twentieth century. The packaging is thus designed to meet these needs by providing ready-to-eat products, which can be purchased in single portions, suitable for use in the microwave, with resealable packages etc. [8,12]

1.3 Food Packaging Materials

The conventional materials most used in the packaging industry can be grouped into four large families: paper packaging, metal packaging, glass packaging and plastic packaging.

All these materials have established themselves on the market, as shown in Figure 2, thanks to their characteristics that have made them suitable for carrying out the functions required for packaging. This chapter will briefly describe the main advantages and disadvantages, especially from an

environmental point of view, of these conventional materials with a focus on plastics, made necessary considering the great gap that has raged for years between those who consider them irreplaceable materials and those who instead consider them an environmental disaster. Finally, another class of materials will be discussed, biopolymers, protagonists of our century, which seem to increasingly represent a meeting point between good technical and environmental performance, without forgetting the inevitable impact on human health.

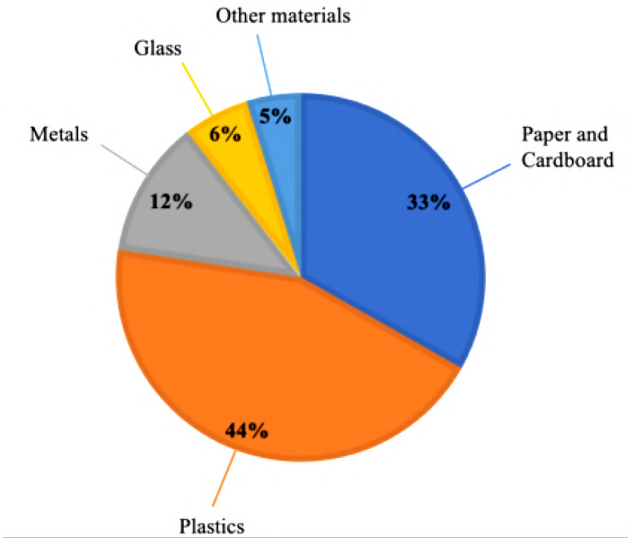


Figure 2: Distribution of packaging materials demand worldwide. Adapted from [13].

1.3.1 Paper and Cardboard

The paper and cardboard packaging sector is estimated to account for 39% of the European packaging market and 31% of the global market, followed by plastics [13,14].

Although paper and its derivatives are a renewable resource, in recent years, more attention are paid to the origin of the wood pulp for paper processing, so more and more forests are managed in a sustainable way, safeguarding biodiversity. The two most important certification programs for sustainable forest management are the Program for the Endorsement of Forest Certification (PEFC) and the Forest Stewardship Council (FSC). The cell wall of wood consists of 40-44% by weight of cellulose, 25-29% of hemicellulose and 25-31% of lignin [6].Through the pulping process, the wood mass is reduced into fibers which are then reformed into sheets. Pulping is accompanied by mechanical, thermal or chemical methods (with sodium hydroxide or sodium sulfate) for the reduction of the lignin content which negatively affects the rigidity and perishable nature of the product. The pulp obtained is then subjected to bleaching to improve the whiteness of the paste using chlorine or hydrogen peroxide, beating and refining to increase the surface of the fibers and therefore

water retention, papermaking through mechanical forming treatments and final treatments such as calendaring or laminating. The bleached pulp papers are mainly used for letters or books, while the raw paper, virgin paper, in unbleached wood pulp are used for food packaging [14]. Of the latter the main and most used are: kraft paper, greaseproof paper, glassine paper and waxed paper [15]. While, paper is generally referred to as cardboard when its grammage exceeds 250 g/m² [6]. The paper sector not only contributes to the gross domestic product but also to environmental pollution due to the high use of water and energy and presents health risks. Disposal methods include recycling, with a limit of 6 or 7 cycles, incineration, landfilling, pyrolysis and composting. As with plastics, the problem of paper disposal is due to the use of mainly chemical additives during the production process to improve the properties, such as mineral oils, dyes, phthalates, polyfluorinated substances, bisphenol A, etc. Unfortunately, recycling does not eliminate these additives, and in some cases increases their migration, especially in fatty foods [16]. In fact, most of the paper packaging is used only in contact with dry foods, while for foods with a high fat or moisture content, the layer in contact with the food is in any case made up of a plastic film.

1.3.2 Metals

The metal packaging sector covers approximately 12-15% of the global packaging market [14,17]. The metals most used in the production of food packaging are not used in pure form but in the form of alloys such as iron and carbon for the production of steel and aluminum alloys. The steel par excellence for contact with food is stainless steel (AISI 304 (EN 1.4301), AISI 316 (EN 1.4401)), with a high resistance to corrosion and inert character thanks to the presence of chromium which in contact with oxygen creates a layer of chromium oxide which passive the surface of the steel. Compared to other materials used for packaging, stainless steel has high costs, therefore it is mainly used for containers for repeated use and equipment in the food sector. Other types of steel used in this sector are coated steel, such as tin and tin-free steel, which are cheaper, recyclable but require surface coatings to make them inert towards food. Finally, aluminum is a highly versatile material for packaging, widely used in laminates for its barrier properties against moisture, oxygen and light superior to that of any laminated plastic material. It is mainly used in the form of thin sheets, trays and lids as well as cookware. Aluminum also tends to passivate itself, but still needs to be chemically anodized to make the surface oxide layer thicker and more homogeneous [18–20]. Metals owe their strengths to excellent barrier properties, malleability, high-temperature resistance, and high recyclability. However, metals are not inert materials for food, therefore they present health risks associated with the migration of heavy metals such as cadmium, mercury, nickel, or bisphenol A, as well as being highly reactive to oxidative phenomena and therefore to corrosion. For both reasons, they require the use of protective coatings [21], which are generally made up of synthetic resins that

create a surface film that is waterproof and resistant to the most aggressive environments, such as in the case of acidic foods. These resins can be based on natural gums and semi-drying oils or based on vinyl lacquers in solvents, phenolic lacquers or epoxy resins.

1.3.3 Glass

Glass has always remained a competitive material in packaging systems and, on the global market in this sector, it covers about 6% [17].

The main component of glass is silica, obtained from sand, melted at high temperatures (900-1600 °C) and subsequently mixed with other elements according to the physical and chemical properties of interest, for example sodium and potassium carbonate, calcium and magnesium, alumina and boron [1,6]. The wide use of this material is to be found in its physical, chemical, optical and thermal properties. Transparency, for example, allows to see the content quality and represents a big advantage for the buyer as well as for the producer. However, it is possible to add color additives [22]. The impermeability is essential to ensure total protection from external atmospheric agents, oxygen and possible contamination. The glass also has a high heat resistance, making it suitable for sudden but gradual temperature changes such as cooking in the oven or in the microwaves [5]. The amber glass also offers complete protection from the penetration of UV rays which act by degrading the food. Furthermore, although its amorphous structure makes it subject to breakage as a result of impacts, the glass has a high load resistance value, which makes it easy to handle during filling and use. The high resistance to internal pressure also makes this compound suitable for the packaging of carbonated drinks [1]. One of the greatest advantages of glass lies in its inert character so it does not react with food, thus allowing safe storage. Finally, it has significant environmental advantages, in fact, the ability to reuse and recycle them has allowed significant economic savings as well as a lower environmental impact [23].This advantage is most noticeable in the local market, i.e., in the distribution of beverages within a radius of 100 km from the production site and with returnable vacuum [24]. As for disposal, glass can be sent for recycling or landfill disposal. More and more countries are adopting recycling systems, up to 50 cycles, as this allows to significantly reduce the potential impact on global warming due to the amount of carbon dioxide emitted during processing [23].

1.3.4 The plastics dilemma

The use of disposable plastic packaging materials has globally grown enormously over the years. In fact, global plastic production increased from 2 million tons in the 1950s to 359 million tons in 2018 as reported in Figure 3 [25].

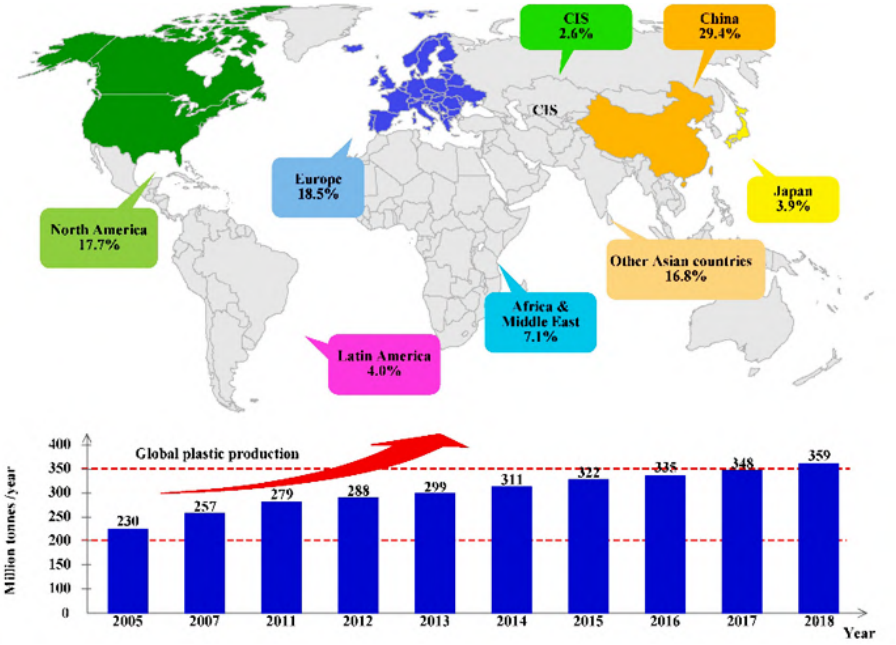


Figure 3: Distribution of plastics globally produced [25].

The main plastic materials used in packaging are generally obtained through the extrusion of thermoplastic polymers including:

- **Polyethylene (PE):** a polyolefin obtained from the addition polymerization of ethylene gas under conditions of high temperature and pressure. It is the most used and affordable polymer; in fact, it is considered as the "workhorse" of the flexible film industry [26]. It is a resistant, heat-sealable material, with a rather low melting temperature (120 °C), with good moisture barrier properties, but poor barrier properties to carbon dioxide, oxygen and fats. However, it can be easily coupled or laminated with other polymers to increase its performance [27]. Two variants widely used in food packaging are high-density polyethylene (HDPE) and low-density polyethylene (LDPE). The first is used for bottles of milk, fruit juices, as well as for linings of cereal boxes. It has excellent resistance to impact, tear, and tensile strength as well as high stiffness, hardness and chemical resistance; the second is used to produce flexible and transparent plastic bags and water bottles and offers a good barrier against moisture [28,29].

- **Polypropylene (PP):** a polyolefin obtained from the addition polymerization of propylene under high temperature and pressure conditions using Ziegler-Natta type catalysts. It is used for both flexible and rigid packaging and thanks to its melting temperature (160 °C), it is suitable for packaging to be used in the microwave. In addition, it has good heat-sealability and transparency. Polypropylene is a chemically inert polymer and it constitutes a good barrier to water vapor and fats [6,26,30].
- **Polyethylene terephthalate (PET):** a condensation polyester obtained from esters and from the reaction between a carboxylic acid and an alcohol. It is suitable for use in sterilizable packaging, for products to be boiled or heated in ovens thanks to its high melting temperature (260 °C). Furthermore, the films made of PET remain flexible even in freezing conditions [26,31].
- **Polyamide (PA):** polymer known by the commercial name of nylon, it is obtained from the condensation polymerization of a diamine and a diacid. It has properties and applications similar to those of PET [26]. It is more expensive than the aforementioned polymers but has better properties [6].
- **Polyvinyl chloride (PVC):** obtained from the addition polymerization of vinyl chloride, this rigid plastic is one of the main materials used in the packaging of food products in Western Europe. It has high hardness and resistance to chemical agents and fats but it requires the use of plasticizers to decrease its fragility with a consequent decrease in its gas barrier properties. Furthermore, it has a low melting temperature (80-90 °C), which facilitates its heat-sealing [6,26,32].
- **Polystyrene (PS):** is a styrene addition polymer, rigid, which has low gas barrier properties and is therefore used for fresh products, in the form of egg containers, fast-food trays, and trays for meat, fish, and poultry. It is often used in its expanded form, which is an easily moldable foam that has good performance in terms of thermal insulation [26]. The melting temperature is relatively low (70-100 °C) [6,33].

To improve the properties of these polymers, the use of additives is almost indispensable [34,35]. The main additives aimed at improving the properties of plastic packaging include:

- Stabilizers: generally based on calcium, zinc, and tin, they prevent the deterioration of synthetic polymers.
- Plasticizers: organic esters of phosphoric acid, fatty acids, and glycerol serve to make polymers such as PVC less fragile.
- Surface property modifiers: these additives are extremely important to keep the surface properties of plastic packaging intact. The most commonly used additives include: anti-drip, anti-fog and anti-blocking agents.

- Dyes: carbon black, red iron oxide and white titanium dioxide. The use of these additives, however, presents an uneven distribution within the polymeric film. For this reason, liquid dyes dosed directly in the molding machine are preferred.

The rapid and inexorable development of these materials of fossil origin was due, first of all, to the undeniable properties that have, such as economy, flexibility, lightness, the possibility of making products with various levels of transparency, color, as well as easy heat sealing, heat resistance and barrier properties. Secondly, plastics have been able to respond to the needs of modern society, increasingly looking for comfortable, easy-to-use products with attractive designs that fit into today's hectic lifestyle with single-portion, light and resistant food packaging. These advantages of plastic have created a real dependence on both consumers and industries.
A major limitation in the use of plastic packaging is the high environmental impact that this waste is having on the ecosystem [36–40]. In fact, about half of the global packaging market is dominated by plastics. As an obvious consequence, as reported in Figure 4, the sector that contributes most to the production of plastic waste is the packaging sector.

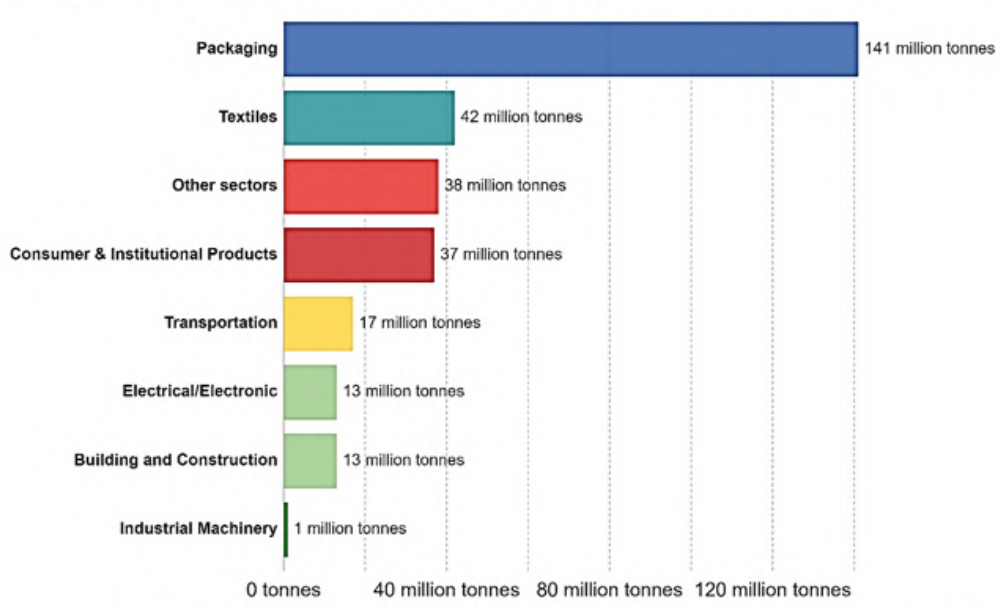


Figure 4: Plastic waste production by sector [41].

Although attempts are made to overcome the problem of disposing of plastic waste with methods such as recycling and incineration, it has been observed that, in every way, plastic waste and its disposal cause serious problems not only for the environment, but also for health. In fact, the

production processes and the disposal of these materials have as their main consequence the production of greenhouse gases, in particular CO_2, with an annual growth trend reported in Figure 5.

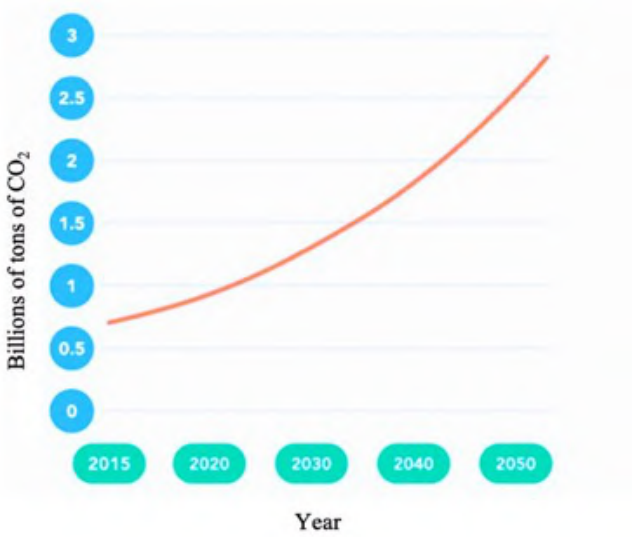


Figure 5: Carbon dioxide emissions from plastics production and combustion [42].

In addition to CO_2, toxic gases are also produced which include substances such as dioxins, furans, mercury. The big problem of disposal is the poor management of this, the lack of collection or correct handling practices means that the packaging ends up in landfills if all goes well, otherwise it remains abandoned in nature, in water, contaminating the oceans, also involving the growing problem of microplastics [43,44]. In Figure 6 two very explanatory images of the serious environmental situation are shown.

Figure 6: Pictures of the impact of plastic packaging on the environment (https://www.focus.it/temi/isole-di-plastica).

The European Commission has adopted a circular economy approach by setting the goal of achieving the recycling of 55% of plastic packaging by 2025 and total recycling by 2030 [45]. In fact, landfill composting of plastics is practically impossible to take into consideration as the degradation rate is about 1% of the mass in 100 years. Aerobic or anaerobic degradation produce significant emissions of carbon dioxide and methane respectively, becoming unmanageable in landfills [46]. Recycling, for many, is the most environmentally friendly and safest way to solve the plastics problem. But, to date, only 14% of all plastic is recycled and in addition, it is then used for applications other than packaging [47]. Recycling can be carried out mechanically or chemically for non-separable multilayer films [48]. In any case, recycling can lead to the formation of new compounds, including toxic ones, which pose a safety problem linked to the possible migration of these on food [49]. Furthermore, the EFSA (European Food Safety Authority) only admits PET as a recycled plastic that is safe for contact with food, as other plastics are rarely used as mono-materials but in the form of coupled as well as enriched with additives as already mentioned [50].

All this has led to a growing interest in the use of bio-based plastics produced from renewable resources, such as bio-PET, bio-PE, bio-PP, produced from biomass, which in any case remain non-biodegradable just like relatives of petroleum derivation [43,51], but they allow to reduce the carbon footprint at least in the production phase [52]. To increase biodegradability, many researchers are focusing on making polymeric blends between plastic and biodegradable natural polymers [53–56]. Finally, to understand even better the dilemma of plastics, it is necessary to consider that their total replacement would, to date, be impossible considering the economic side of the matter. In fact, in Europe alone, the plastics market bills around 360 billion euros a year and supports 60.000 companies [52]. Therefore, the road to the zero-waste approach will have to be driven by consumer awareness and correct disposal practices, as well as by legislation and incentives for companies to adopt biodegradable materials, also considering the rising price of oil.

1.3.5 Biodegradable polymers and biopolymers

Before entering into the merits of this paragraph it is necessary to report the definition of some often-confused terms, following the UNI EN 13432: 2002 standard.

- **Degradable:** term referring to materials that can be disintegrated and fragmented due to specific environmental conditions that cause changes in the chemical structure of the material. Degradation occurs in all materials but with different timing [57].
- **Biodegradable:** term applied to materials that decompose at least 90% into carbon dioxide, water, methane, biomass and organic compounds, by the action of microorganisms such as fungi, molds and bacteria within 6 months. Since biodegradability does not depend on the source but on the

chemical composition of the polymer, this can be biodegradable whether it derives from natural and renewable sources or from fossil sources [6,58].

- **Compostable:** term referring to materials whose organic part is degraded relatively quickly (less than 3 months), by a microbial pull and in a humid and oxygenated environment without producing eco-toxic effects. Composting products are CO_2, water, inorganic compounds and biomass. A biodegradable material is not necessarily compostable, while the opposite is true [6,59].
- **Bio-based:** term referring to polymers derived from renewable sources. A bio-based polymer is not a sustainable polymer in itself, this depends on the starting source and the production process. In general, not all bio-based polymers are biodegradable (such as bio-PE and bio-PA), and not all biodegradable polymers are bio-based (such as, for example, polycaprolactone). There are also polymers that are both bio-based and biodegradable, such as polyhydroxyalkanoates. Fossil resources also fall, in a certain sense, into the bio-based category and derive from renewable sources, but the times of consumption and regeneration of biomass are highly unbalanced, in fact biomass turns into oil in more than a million years, for this reason they are generally excluded from this category which instead presupposes comparable consumption and regeneration times [6,60]. In Figure 7, an example of bio-based packaging material cycle adopting a circular economy approach is reported:

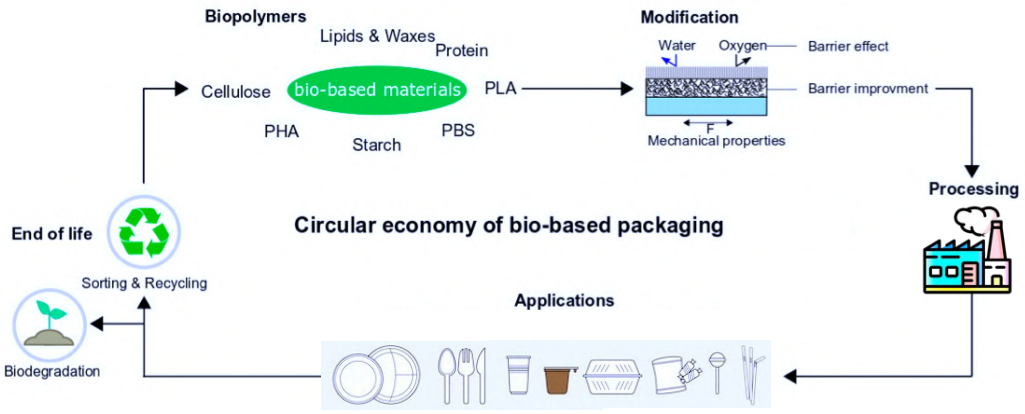


Figure 7: Bio-based packaging material cycle in circular economy perspective. Adapted from [61].

- **Biopolymers:** are defined as natural polymers produced by the cells of living organisms. They belong to the category of bio-based materials and represent a valid alternative to polymers of fossil origin, so much so that they fall within the Sustainable Development Goals 6, 9 and 12 defined by ONU in the 2030 Agenda [62]. Biopolymers can be classified into three categories based on their origin and production mechanism: polymers directly extracted from biomass, biodegradable

polymers synthesized from renewable or oil-based monomers, polymers produced directly by microorganisms. In the next paragraphs all these categories will be discussed, reporting a description of the main biopolymers of interest for the food packaging sector and, in particular, the biopolymers used in this project, zein, chitosan and a non-bio-based polymer will be explored, however biodegradable, polycaprolactone.

1.3.5.1 Polymers directly extracted from biomass

These polymers are extracted directly from agricultural waste and biomass of plant and animal origin. Polysaccharides and proteins belong to this category.

- **Polysaccharides**

They are organic molecules with a high molecular weight made up of long chains of monosaccharides linked together by glycosidic bonds. Being obtained from sources of natural origin, polysaccharides are generally non-toxic and biodegradable, which makes them suitable materials for sustainable development.

- **Cellulose:** it is the most abundant natural polymer on earth, consisting of a linear chain that can contain from many hundreds to more than 10 thousand units of D-glucose joined together by β (1 → 4) glycosidic bonds. Cellulose is one of the main components of lignocellulosic biomass, together with lignin and hemicellulose, and constitutes between 35 and 50%. It is an inexpensive raw material but difficult to use due to its hydrophilic nature, insolubility and crystalline structure. The solvents used to dissolve cellulose must be able to break the hydrogen bonds, in order to separate the polymer chains from each other, an example of a cellulose solvent is N-methylmorpholine-N-oxide [63]. Therefore, natural cellulose being insoluble in water and unable to be thermally processed, since it has a lower decomposition temperature than the melting point, is converted into its derivatives to make it more processable [64]. Among the derivatives of interest for packaging: cellulose acetate (CA), a cellulose ester that has excellent processability, stability at room temperature and transparency, deserve to be mentioned; Cellophane, a material that has excellent mechanical properties but is very hydrophilic and, therefore, sensitive to moisture and, moreover, cannot be heat-sealed [65].
- **Starch:** it is cheap and abundantly available from sources such as potatoes, corn, wheat, rice, tapioca etc. It is composed of amylose, a linear and crystalline polysaccharide, and amylopectin, a branched and amorphous polymer. The proportions between the two compounds vary approximately between 10-20% of amylose and 20-80% of amylopectin, depending on the source from which it derives; this gives the material different mechanical and biodegradability properties [66]. As a packaging material, however, starch alone does not form films with adequate

mechanical properties unless it is first treated by plasticization, mixing with other materials, genetic or chemical modifications, or a combination of the aforementioned treatments [67].

- **Alginate:** it is an unbranched linear polysaccharide present in brown algae and marine algae such as *Laminaria hyperborea*, *Ascophyllum nodosum* and *Macrocystis pyrifera*. Alginate can be converted into its salts, of which sodium alginate is the main form currently used. These polymers are made up of two different monomers in varying proportions and have a high molecular weight. The monomers that constitute it are β-D-mannuronic acid and α-L-guluronic acid linked in α- or β-1,4 glycosidic bonds. Alginates are often used as stabilizers in emulsions, thickeners, and binding agents. Thanks to its biodegradable and non-toxic properties, it finds application as a material for food packaging in the production of edible films [68,69].
- **Carrageenan:** comes from an alga called carragheen or Irish moss. There are three basic types of carrageenan: kappa (κ), iota (ι), and lambda (λ). λ-type carrageenan produces viscous but non-gelling solutions, κ-type carrageenan forms a brittle gel, while ι-type carrageenan produces elastic gels. In the food industry, carrageenan is used to make edible films and coatings, and of the three forms mentioned above, k-carrageenan is the most widespread. Carrageenan can also be added with other polymers to produce fresh food coatings as they reduce moisture losses, decrease gas exchange and prevent discoloration [70].
- **Pectin:** it is an amorphous, white, and colloidal carbohydrate with a high molecular weight present in ripe fruits, especially apples and currants. Pectin substances are found in the primary cell walls and are normally associated with the structures of cellulose, hemicellulose, and lignin. Pectin is mainly composed of a linear chain of galacturonic acid monomers. The composition of pectin can vary according to the botanical source and the main sources of extraction of pectin are apple peels, pomace and citrus fruits, which are by-products, for example, of the juice industry [71]. Due to its rheological properties and non-toxicity, pectin is widely used in the food industry as a stabilizer, thickener, texturized, and emulsifier. Edible coatings produced from pectin and its derivatives have recently been suggested for applications in the food sector due to their excellent barrier to oxygen but not to humidity, oil barrier, aroma retention, and good mechanical properties. Edible pectin films can be added with various antimicrobial substances in order to confer an antimicrobial action that increases the shelf-life of the product and reduces the risk of growth of pathogens on food surfaces [72].
- **Chitin and Chitosan:** they are polysaccharides very present in nature, especially in the exoskeleton of crustaceans, insects, fungi, and snakeskin. Chitin is the second most abundant polymer after cellulose and is used in many fields: the production of surgical sutures, in the ophthalmic field, to speed up wound healing, in the production of controlled-release drugs, and in packaging for its antibacterial properties [73]. Chitosan is a hydrophilic linear copolymer composed of D-glucosamine and N-acetyl-D-glucosamine, linked by β (1-4) glycosidic bonds

and can be obtained from the deacetylation of chitin. Chitin extraction can be done chemically or biologically. The chemical route requires the use of solvents such as strong acids (acetic acid, nitric acid, formic acid, sulfuric acid, etc.) for the elimination of minerals from the biomass, basic treatments for breaking the chemical bonds between chitin and proteins, and discoloration by hydrogen peroxide or acetone. The biological way instead exploits the production of enzymes and organic acids thanks to microorganisms [74]. The production steps of chitin and chitosan are schematized in Figure 8. Chitosan, as mentioned, is then produced by the deacetylation reaction by alkaline hydrolysis.

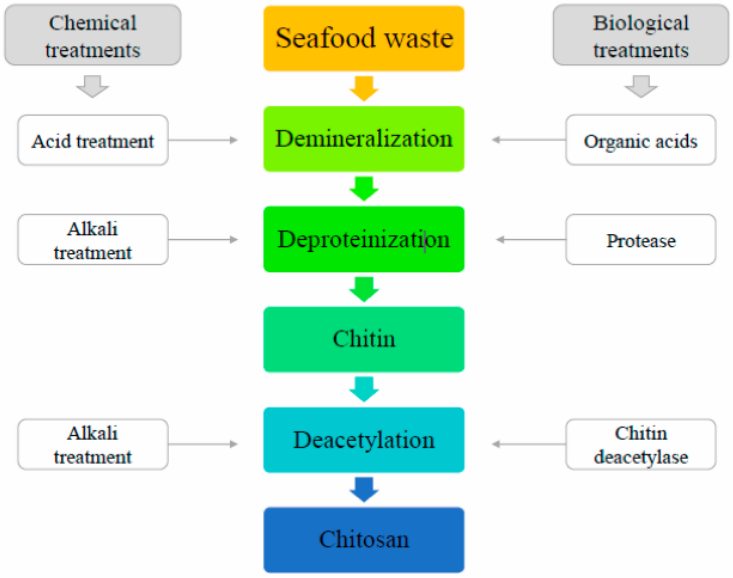


Figure 8: Chitin and chitosan production steps [74].

Chitosan has been studied for many years as it is the only natural polymer to possess antimicrobial properties against viruses, bacteria and fungi [75]. Chitosan is insoluble in water but very soluble in acid solutions. It has a variety of promising pharmaceutical uses and is currently being considered a new carrier material in drug delivery systems [76]. Chitosan is also used in food packaging for its antimicrobial activity and non-toxicity even if there is no scientific data yet regarding any allergenic effects due to its source of origin. Chitosan-based antimicrobial packaging has the potential to inhibit the activity of pathogenic microorganisms that contaminate food and therefore has the ability to extend shelf-life, improving the quality of packaged food. It also has good barrier properties to gases and aromas in the absence of humidity. These characteristics, combined with the ease of film formation make chitosan a good choice for food

packaging applications [77]. Furthermore, the ability of some insects, such as *Hermetia illucens*, to produce this polymer, if raised on waste such as crustaceans, makes it a real alternative resource to synthetic polymers, also considering the revaluation that is carried out on waste [78].

- **Proteins**

They are natural polymers capable of forming stable three-dimensional amorphous structures, mainly through non-covalent bonds. The monomeric units that constitute them are amino acids, linked to each other through a peptide bond, which is a bond between the amino group of one amino acid and the carboxylic group of the other, the proteins can be divided into proteins of animal origin and proteins of vegetable origin.

- **Casein:** it is a milk-derived protein consisting of four amphiphilic sub-fractions: αS1-casein, αS2-casein, β-casein, and κ-casein. The dry matter of bovine casein micelles is about 94% protein and about 6% low molecular weight compounds generally known as colloidal calcium phosphate (CaP). The amphiphilic nature of casein makes them when dispersed in aqueous solutions, easily self-assembling in stable micellar structures, where the four phosphoproteins mentioned above are tightly held together through hydrophobic interactions and by the connection of colloidal CaP. The processing of casein added with suitable plasticizers, at temperatures around 80-100 °C, allows for obtaining materials with variable mechanical properties. Casein does not dissolve in water directly but tends to absorb 50% by weight in a day. Today it is used as a glue for labels due to its excellent adhesive properties [79].
- **Gluten:** another protein used for the production of biopolymers is gluten, a food complex consisting essentially of the main reserve proteins of the kernels of some cereals: gliadin and glutenin; it is obtained by mixing the flour of some cereals with water. Mechanical gluten treatments lead to the formation of disulfide bridges between the residues of the amino acid cysteine, which is relatively abundant in gluten. The resulting mixture is presented as a viscoelastic network, capable of combining cohesion and elasticity. The processability of gluten is difficult and requires the reduction of disulfide bridges with suitable agents. In the presence of these agents, the processing temperatures are between 70 °C and 100 °C. The properties that gluten-based biopolymers possess are: very high gloss values and good resistance in the water, because they do not dissolve in it, but tend to absorb it, leading to swelling phenomena [80].
- **Soy Proteins:** they are a complex blend of proteins with very different molecular properties. Most soy proteins are globulins (90%). The soy proteins used in the food industry are classified as soy flour, concentrated or isolated, based on the protein content. Soybean meal contains 50-59% protein and is obtained by grinding defatted soy flakes. Soy Protein Concentrate contains 65-72% protein and is obtained by aqueous liquid extraction or acid leaching process. The soy protein isolate contains more than 90% protein and is obtained by aqueous or alkaline extraction followed

by isoelectric precipitation. Soy proteins are widely used in the food industry thanks to their functional properties such as cohesion, adhesiveness, pulp and fiber formation, and solubility. Soy is also used for the formation of edible and biodegradable films. Soy protein films are flexible, smooth, transparent, and clear compared to other vegetable protein films. These films are generally not very water resistant and are produced by drying thin layers of film-forming solutions produced by solvent casting [81].

- **Collagen:** it is a fibrous protein found mainly in specific parts of invertebrate and vertebrate animals, such as skins, bones, tendons and connective tissues. It is composed of various polypeptides, which mainly contain glycine, hydroxyproline, lysine and proline. More than 22 different types of collagens have been identified in the human body so far, the most common being those between type I and type IV. Type I collagen is the most abundant protein in mammals and the most studied. Collagen is a hydrophilic protein; therefore, it swells in polar liquids with high solubility parameters. Collagen is particularly soluble in acidic aqueous solutions and can be processed into different forms such as sheets, fibers, sponges, foams, injectable viscous solutions and dispersions. As for the food industry, collagen is used for its properties such as film-forming ability, biocompatibility, and resistance to organic solvents, short-term biodegradability, and non-toxicity. Collagen films are used as a barrier membrane to protect against the migration of moisture, oxygen, and solutes and ensure the structural integrity and vapor permeability of products [82]. However, the main use of this biopolymer remains in the biomedical, pharmaceutical and nutraceutical fields.
- **Gelatin:** it is a water-soluble natural protein produced by the hydrolysis of collagen. Edible gelatin films are made by dissolving gelatin in hot water, melting, and subsequently drying in the oven. Edible gelatin-based films are thick, have a high protein content and good mechanical properties, but not high water vapor permeability [83].
- **Zein:** it is a hydrophobic protein extracted from corn, the most produced cereal in the world. The protein content of corn varies between 6% and 12% on a dry basis, according to the different varieties, while the components most present are starch and water, and a small part of oil is present mainly in the germ. About 75% of the proteins are contained in the endosperm, while the remainder is contained in the germ. Four main classes of proteins are identified, which differ on the basis of their solubility in selected solvents. Zein belongs to the class of prolamins and is soluble in alcoholic solutions, such as hydroalcoholic mixtures based on ethanol, of which the ternary phase diagram is shown in Figure 9.

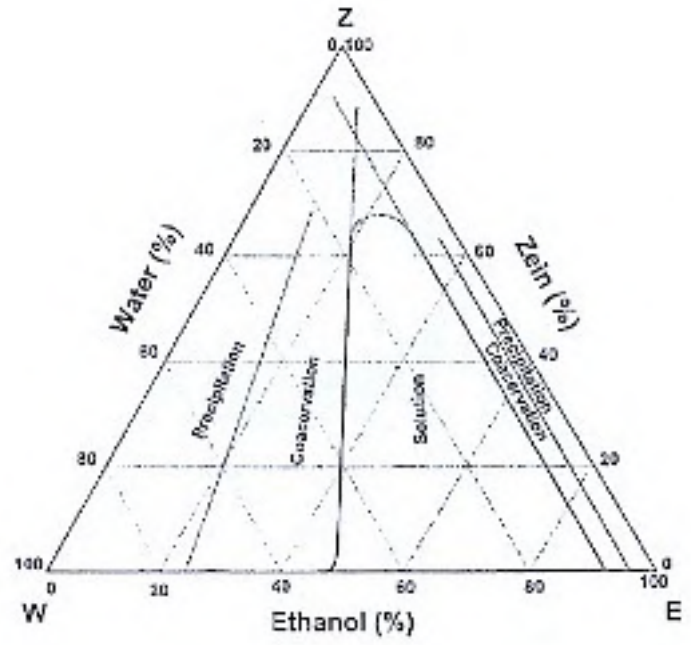


Figure 9: Ternary phase diagram of zein, water and ethanol at room temperature [84].

It is also soluble in other solvents such as propylene glycol, formic acid, acetic acid, aqueous solutions of acetone, methanol, isopropanol, chloroform, etc. It is particularly rich in glutamine, leucine, proline, and alanine. The high amount of non-polar amino acids presents and the lack of acidic or basic amino acids are responsible for the insolubility of zein in water [85].

Pure zein is odorless and colorless, but the marketed zein is yellow in color and contains a low percentage (less than 2%) of non-protein solids and oils [84]. It is mainly produced through two processes: CPC Process, Nutrilite Process [85], of which the process diagrams are reported in Figure 10 and 11 respectively.

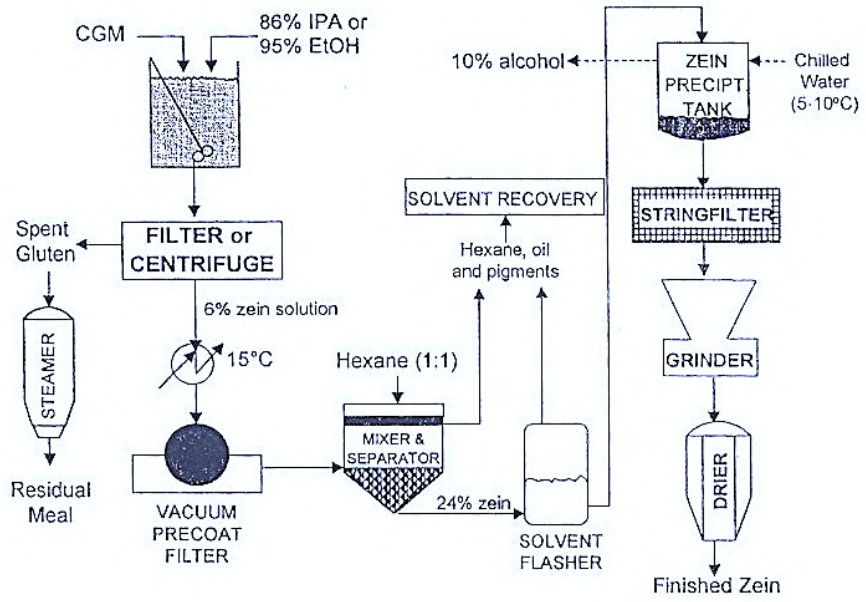


Figure 10: CPC Process for the extraction of zein [85].

In both processes, the main solvent used to extract zein from corn is an aqueous solution of 88% isopropyl alcohol (IPA). In the CPC process, 95% ethanol is also used, with subsequent treatment in hexane for the removal of impurities such as lipid fractions. In the Nutrilite process, on the other hand, a second extraction is carried out in the aqueous IPA solution to remove the oils.

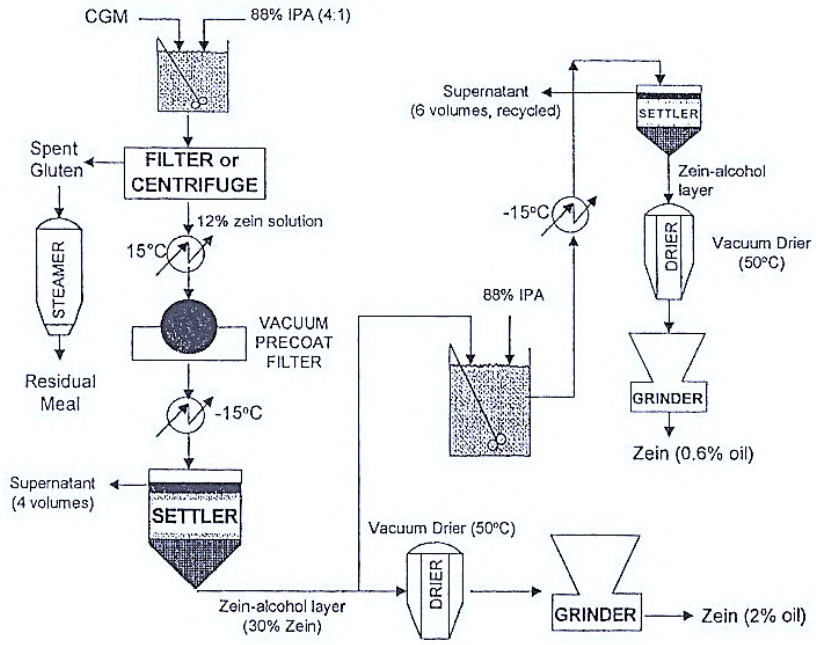


Figure 11: Nutrilite Process for the extraction of zein [85].

Zein, however, has a thermoplastic behavior with remarkable flexibility and adhesion properties, which can be reached thanks to the addition of various plasticizers, such as fats or esters. Thanks to its well-known film-forming capacity, this protein is widely used in the food packaging sector thanks to the production of films, mainly with casting and extrusion techniques [86]. The films obtained are generally fragile and, therefore, it is necessary to use plasticizing agents such as glycerol, polyethylene glycol, fatty acids [87]. The possibility of also making completely edible and therefore zero-waste coatings makes it a biopolymer of great interest for 21st-century research [88]. Furthermore, being insoluble in water and deficient in terms of amino acid profile, it is not intended for human consumption, therefore its use for the production of films for food packaging does not conflict with human nutrition by satisfying the Principles of Green Chemistry and it is one of the few proteins approved by the FDA.

1.3.5.2 Biodegradable polymers synthesized from renewable or oil-based monomers

This category of polymers is a cross between plastics and biopolymers, in fact they are polymers synthesized with conventional methods starting from monomers that can be obtained from both renewable and fossil sources. In any case, they can be included in the category of biopolymers as biodegradable polymers.

- **Polylactic Acid (PLA)**: an aliphatic polyester polymerized starting from lactic acid monomers (2-hydroxypropanoic acid). The monomers are in turn produced through the bacterial fermentation of carbohydrates, renewable resources. It is a versatile, recyclable and compostable polymer, with high transparency, high molecular weight, good workability and good resistance to water solubility. Typically, commercialized PLA is a copolymer consisting of a mixture of its two enantiomeric forms (L - (+) and D - (-) lactic acid). Depending on the ratio of the enantiomers, the properties of polylactic acid can vary considerably from semi-crystalline to amorphous [89]. Polymers derived from lactic acid can be synthesized by polymerization by direct condensation of lactic acid, obtaining the so-called poly-lactic acid, or by polymerization with the opening of the ring through the intermediate product lactide, obtaining the so-called poly (lactide). To be produced on a large scale, polylactic acid must have adequate thermal stability, in order to maintain the molecular weight and properties and avoid degradation, mainly caused by hydrolysis and oxidation phenomena. The biodegradability characteristics of PLA and its high resistance to water vapor have led to its wide application as a primary packaging already on the market, not only for food but also for personal hygiene products [90].

- **Polycaprolactone (PCL):** poly(ε-caprolactone), also known as polycaprolactone (PCL), is a hydrophobic biodegradable synthetic linear aliphatic polyester. PCL, which polymer chain consists of repeated hexanoate units, can be synthesized following two different approaches. The first method refers to the 6-hydroxyhexanoic acid condensation and ring-opening polymerization (ROP) of ε-caprolactone schematized in Figure 12.

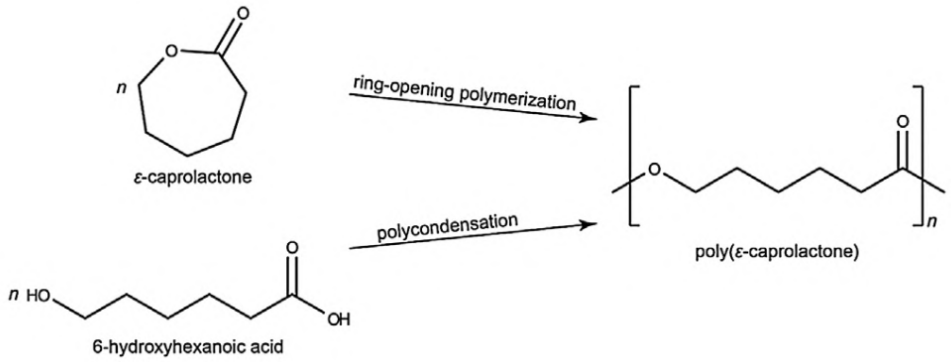


Figure 12: Synthesis of poly(ε-caprolactone) from ε-caprolactone or 6-hydroxyhexanoic acid [91].

The second method is the most widely used since it gives polymer with lower polydispersity and higher molecular weight. There are four different mechanisms for ring-opening polymerization: anionic, cationic, monomer-activated, and coordination-insertion ROP (Figure 13). Metal-based, organic and enzymatic catalysts can be used in ROP. One of the most widely used catalysts, due to its high efficiency and low toxicity, is stannous (II) 2-ethylhexanoate [92].

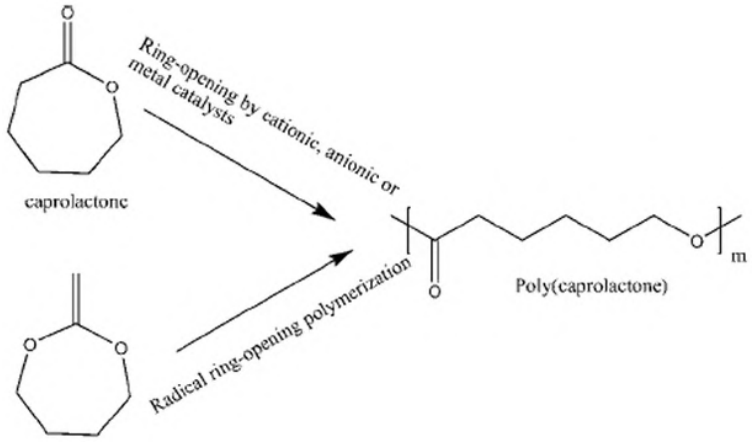


Figure 13: Synthesis of PCL by radical ring opening polymerization [92].

In terms of chemical and physical properties, PCL is characterized by a molecular weight that can range from 530 to 630,000 Da, it has a rather low melting point, which varies between 59 and 64 °C, and its glass transition temperature is about -60 °C. PCL is soluble in many organic solvents, for example, in chloroform, dichloromethane, benzene and toluene, but not in water, alcohols, petroleum ether and diethyl ether [92]. In addition to being biodegradable and compostable [93], PCL is compatible with a wide variety of polymers, it melts easy, and it is relatively low cost. The good processability of PCL also enables to obtain a variety of structures and shapes. In fact, PCL can be molded into microspheres, microcapsules, nanoparticles, pellets, implants, filaments and films. In addition, PCL has been approved by the Food and Drug Administration (FDA) for use in human organisms and finds application in the biomedical field in drug delivery systems, absorbable sutures, and scaffolds [94,95].

1.3.5.3 Polymers produced directly by microorganisms

This class of biopolymers certainly represents a great innovation in the polymeric world. Producing biodegradable polymers starting from bacteria fed with biomass inside fermenters opens the way to new, greener horizons. But, to date, an industrial scale-up is still difficult, especially for polyhydroxyalkanoates.

- **Polyhydroxyalkanoates (PHA):** represent a class of semicrystalline polyesters synthesized by microorganisms (*Cupriavidus necator*, *Alcaligenes latus*, *Pseudomonas putida* and *Pseudomonas mendocina*, *Shewanella oneidensis* and *Aureispira marina*, but also *Escherichia Coli*) [96]. They represent a very broad class of thermoplastic polyesters which includes more than 100 polymers obtained from different starting monomers. The main limitations attributed to the major exponent of this class, the polyhydroxy butyrate (PHB) homopolymer, are due to its high crystallinity and consequent fragility which makes its processability complicated. By intervening on the type of substrate, the culture conditions or by genetic bacteria manipulation [97], it is possible to push the bacteria to produce copolymers of hydroxybutyrate, hydroxy valerate and hydroxy hexanoate in order to lower the degree of crystallinity, which is a fundamental property that influences mechanical, optical, thermal properties and also the biodegradability and depends on the nature of polymer and the production method [98]. PHA can be processed with classic polymer processing techniques to obtain films and sheets, fibers, laminates and coated articles, which can be used in various sectors including food packaging [99].

- **Polybutylene succinate (PBS):** it is a semi-crystalline biopolymer obtained from the polymerization of butanediol and succinic acid which can be produced both from oil-based sources and from renewable bio-based resources through bacterial fermentation on renewable substrates such as glucose and starch. Among its main characteristics, it has a good thermoplastic workability, compared to other common polymers, together with a good thermal and chemical resistance. However, the use of PBS for the manufacture of polymer membranes has been limited due to its brittleness and poor gas impermeability. To overcome this limitation, PBS is often mixed with other polymers such as polyethersulfone (PES) and cellulose acetate (CA); by adding these two amorphous polymers to the PBS, the resulting membranes showed lower crystallinity and higher mechanical strength than unmodified PBS membranes [100].

Biopolymers produced directly from biomass are mostly hydrophilic and crystalline and therefore can present problems in the production phase and offer a fertile ground for research in terms of achieving adequate performance for the final application. The other two categories of biopolymers, on the other hand, have higher costs, especially when compared with those of traditional plastics. The main issues related to the use of biopolymers in food packaging are poor mechanical properties, for example high brittleness, low heat tolerance, high sensitivity to humidity, and cost. However, the possible application of biopolymers is wide and includes rigid packaging, coatings, and films. The use of coatings implies their formation directly on the surface of food products, while the films, the object of this work, are flexible structures that are applied after being formed separately. However, it is already possible to find some examples of packaging, even edible, on the market or start-ups launched, especially in the extra-European market, such as Ooho to replace plastic water bottles, Loliware with edible glasses, and Paktin with an edible spray gel, and others (https://www.foodaffairs.it/2022/03/07/il-packaging-commestibile-e-la-nuova-frontiera-della-sostenibilita-nel-food/).

To be competitive materials with respect to petroleum-derived plastics, it is important that these new packaging solutions are economically viable so that they can be easily integrated into current industrial production processes [101]. But, to compare the sustainability of these materials with respect to those of fossil origin, it is necessary to carry out an assessment not only economic but also of the impact on the environment, the carbon footprint, food safety, the different management of packaging waste (which could, in some cases, become zero-waste) and finally, the possible reduction of food waste volumes if these biopolymers were used to create those systems that in a certain sense represent "packaging 4.0", i.e., smart packaging, which will be subject of the next chapter.

1.3.6 Innovations in food packaging field

The contents of this section have already been published in a review:

- E. Drago, R. Campardelli, M. Pettinato, P. Perego. Innovations in Smart Packaging Concepts for Food: An Extensive Review. *Foods,* 2020, 9, 1628. Doi: 10.3390/foods9111628.

It has been seen so far that traditional food packaging systems have been attributed a passive role in protecting products from the external environment. For some years now, however, the way of conceiving food packaging has been completely revolutionized. Innovation in this field can be summarized using some particular terms which are: active, intelligent, smart, etc., [102], all terms to indicate that packaging becomes dynamic, playing a leading role in the preservation of food and in maintaining the nutritional and organoleptic qualities, safety and quality of products throughout the distribution chain [103]. To perform these functions, the new packaging systems have to interact with the product itself, again upsetting another fundamental principle of traditional packaging: inertia towards food. To better understand how this interaction can take place, it is necessary to report some definitions of the terms mentioned above, which are often used erroneously in a unique way when instead they indicate very different categories of systems [104].

Regulation 450/2009 of the European Commission defines all those systems as active materials "designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food" [105]. The main objective of these systems is to intervene in the conservation status of a food, preventing premature biological and chemical contamination. Also in the same European Commission regulation, smart packaging is defined as "materials and articles that monitor the condition of packaged food or the environment surrounding the food" [105]. The main objective of these intelligent systems is therefore to provide, in a constant manner, information on the conditions of the product, understood as the set of food and packaging products, in order to ensure a safer and more efficient management of the distribution of products [106].

Therefore, as represented schematically in Figure 14, the main difference is that active packaging acts directly on food or the environment surrounding food with the aim of increasing and protecting the shelf-life, while intelligent packaging does not interact with the food product but it monitors and communicates information about the conditions and alterations of the packaged product [107]. These two innovative ways of conceiving food packaging can lead to a unique complex system that performs both functions, called a "smart packaging" [106].

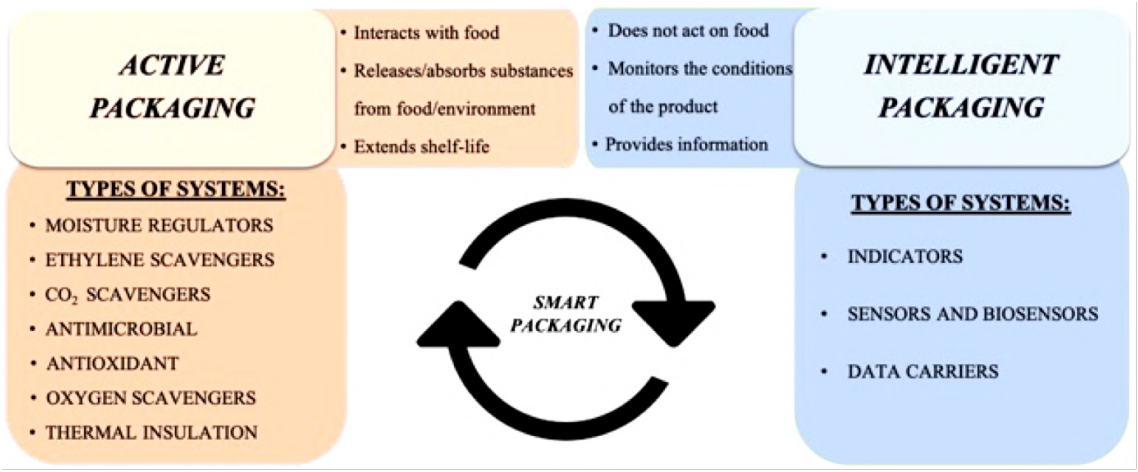


Figure 14: Schematization of Active and Intelligent packaging types and properties.

It is also important to underline that these innovative food packaging systems already play an important role in the extra-European market, especially in the USA, Australia, and Japan, while they are still rare if not absent in the European one [108], even in due to the stricter European legislation, for which reference should be made to paragraph 1.5. The main types of active and intelligent packaging will be discussed in the following paragraphs. In particular, the types of systems that have been the subject of this work will be analyzed in greater detail: active packaging with antimicrobial, antioxidant and thermal insulation action.

1.3.7 Active packaging and shelf-life

There are many different types of active food packaging that perform many different actions. Generally, it is possible to distinguish two macro-classes of active packaging:

1. Non-migratory active packaging: those used as external components to the polymeric packaging material, therefore in the form of bags [109], or pads [110], to be inserted inside the primary packaging, mainly placed in contact with the headspace of the packaging and not with the food itself.
2. Migratory active packaging: all those systems in which the active compound is an integral part of the polymeric packaging material. Here, the active ingredient can be added either by surface coating [111], as commonly occurs on an industrial scale, or immobilized on the inner surface of the package by means of post-production treatments of the package, or loaded inside the polymeric material during the phase of production of the packaging itself through various encapsulation and film production techniques [112,113].

The distinction between these two classes, also in terms of positioning of the systems with respect to the product, is well represented in Figure 15, in which the non-migratory systems (scavengers and absorbers) are represented on the left and the migratory ones (antimicrobials and antioxidants) on the right, while Table 1 shows some examples of active packaging already on the market.

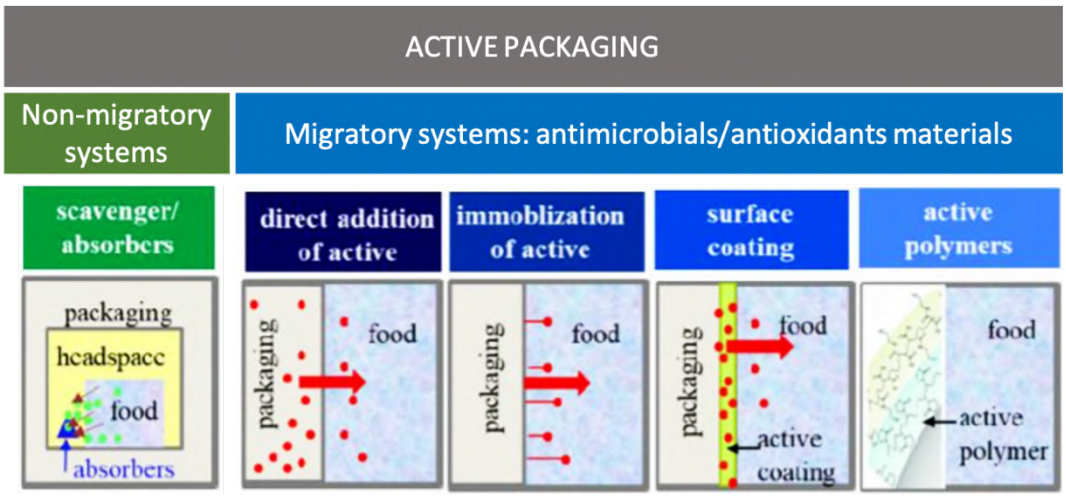


Figure 15: Classification of different types of active packaging systems for food. Adapted from (Wiley, ISBN: 978-1-119-82507-4).

The common factor of these types of active packaging lies in the purpose, which is to preserve and/or extend the shelf-life of foods. The shelf-life of a product represents the period during which a food product can remain in good condition, maintaining the desired physical-chemical and sensory qualities and nutritional values and guaranteeing food safety. Alteration of the shelf-life can occur due to incorrect storage methods, loss of integrity of the packaging designed to protect the food from external degrading factors, such as light, oxygen, humidity, and bacteria. It is clear that every food is destined to perish more or less quickly, but premature spoilage, before the expiration date, leads to the onset not only of problems related to food safety, but also to the serious problem of food waste. In fact, according to the Food and Agriculture Organization of the United Nations (FAO), every year in the world 1.6 billion tons of food, about 1/3 of all food produced for human consumption, are lost or wasted [114]. A waste of such dimensions represents a significant issue from the environmental, social and economic point of view.

With this in mind, active packaging can really act as a prevention system by mitigating the consequences of an alteration in the shelf-life.

Below is a brief description of the types of active packaging, with the exception of migratory ones with antimicrobial and antioxidant action and non-migratory ones with thermal insulation action, for which a separate paragraph has been dedicated as they have been tested for this work. Among the non-migratory active packaging there are:

- **Moisture Absorbers and Regulators:** excess moisture is one of the major causes of food spoilage and, for this reason, it is very important to use absorbers or desiccants. The low permeability of packaging materials to water vapor, sudden changes in temperature, but also the normal breathing of fresh products can cause an accumulation of moisture inside the packaging, which leads to microbial proliferation with consequent loss of product quality. These systems are mainly based on the use of hygroscopic materials, such as activated clays and silica gel, contained in bags placed inside the primary packaging. The most used are those with silica gel, which can absorb water up to 35% of its own weight [115,116]. Polyvinyl alcohol can also be used, in the form of films placed inside or in an intermediate area of the main packaging [117,118]. If these systems act by reducing the moisture of the headspace of the pack, then they are defined as humidity regulators, in the case of absorbers, these are used to absorb the liquids that form at the bottom of the packs of fresh products with a high activity of the water in the form of patches and sheets [10], but without dehydrating the food product.
- **Oxygen Scavenger:** the sensitivity of food to oxygen often results in changes in appearance and organoleptic properties. The oxygen scavenger systems can be used alone or in combination with modified atmosphere packaging, where part of the atmospheric oxygen present is removed [119,120]. The most used oxygen absorber is based on the oxidation of iron-based compounds contained within oxygen-permeable bags. Thanks to the high absorption capacity of iron compounds, this type of absorber is the most effective on the market [121]. Several experiments are currently underway to incorporate iron compounds that act as absorbers in different types of polymers. In this way, the absorbers can be in the form of labels glued to the inside of the package, seals, or be an integral part of the package itself. Other substances used as absorbers are mainly metal catalysts, salts or enzymes [122].
- **Ethylene Scavenger and Adsorbents:** ethylene is a phytohormone normally produced during the ripening of climacteric plants and which favors the degradation of chlorophyll with consequent deterioration of the product. Ethylene can be removed by absorption systems that trap it or by chemical reactions from scavenger systems. The most commonly used absorption systems are sachets filled with zeolites, silica, clays, or activated carbon [123,124], while scavengers are generally composed of potassium permanganate incorporated in silica gel or in any case inert supports [125]. The silica gel with permanganate is contained in sealed sachets permeable to ethylene, without the possibility of coming into direct contact with the food given its toxicity [126].

- **Carbon Dioxide Scavenger:** carbon dioxide is generally beneficial in food packaging but, an excess can be a signal of undesirable effects. In fact, the microbial activity produces high levels of CO_2, leading to potential undesirable organoleptic changes of food, such as discoloration, off-flavor and tissue breakdown [127]. The main carbon dioxide scavengers are represented by porous sachets with calcium oxide which can react with water by forming calcium hydroxide, which finally reacts with CO_2 producing calcium carbonate [128]. Other absorbers consist of silica gel, sodium hydroxide, potassium hydroxide [116], zeolites or activated carbon [127].

1.3.7.1 Antimicrobials and Antioxidants active packaging

Active packaging with antimicrobial and/or antioxidant activity plays a vital role in the packaging of perishable food products such as meat, fish, poultry, and horticultural products, as these products contain all the nutrients and conditions for potential microbial growth and are highly susceptible to oxidation which, if not slowed down, leads to the rapid deterioration of the food matrix with a reduction in shelf-life, organoleptic changes and consequent increase in food waste [107]. In particular, contamination by pathogens, such as Gram-positive bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Enterococcus faecalis),* Gram-negative *(Escherichia coli*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa* and *Salmonella choleraesuis*), molds (*Penicillium roqueforti* and *Aspergillus flavus)* and yeasts (*Candida albicans* and *Zigosaccharomyces rouxii),* can take place at any point in the supply chain, but it is difficult to monitor from the moment the product leaves the production site and reaches the shelves of consumers. Microbial growth, not always visible to the naked eye, carries serious risks of toxic infections for consumers, and, to date, it is still a widespread problem all over the world [116]. For this reason, active antimicrobial systems together with antioxidant ones are currently the group of active packaging of greatest interest in research and in the food industry, because they have the potential to provide substantial help in maintaining shelf-life and reducing food risk.

The additives used for this type of active packaging can be classified as natural or synthetic depending on the source of origin, but in any case, they must be approved by the FDA for contact with food [129]. Furthermore, some compounds, especially those of natural origin, can have both antimicrobial and antioxidant action, so they will be treated together in this chapter. The compounds of natural origin are the most studied as they allow the re-evaluation of agro-industrial waste often still rich in molecules with high added value that can be used in various fields, from biomedical and pharmaceutical to that packaging, offering a fertile ground for research. This category includes essential oils and natural extracts of plants and spices. Many plants, in fact, are rich in antioxidant substances, in particular, vitamins, phenolic compounds, carotenoids, and polyphenols which act by inhibiting the oxidative reactions of food matrices thanks to the release of hydrogen atoms or

electrons that lead to the formation of a non-reactive and stable radical [130]. Phenolic compounds are also abundantly present in the essential oils of some plants, such as cinnamon, garlic, oregano, thyme, lemongrass, rosemary, etc., and can also have antimicrobial, antifungal, and antiparasitic properties as well as antioxidants, acting on bacteria by inhibiting them, damaging their cell membrane or acting directly on microbial metabolism thanks to their hydrophobic nature which facilitates their penetration into the cell membrane of bacteria which causes their death [131–133]. Usually, essential oils are not used as additives directly in contact with food as their strong organoleptic characteristics can alter those of the products, so their use in packaging materials offers a preferable solution for their use [134]. Furthermore, some studies have reported a possible synergistic action between natural compounds if used as a whole instead of as single isolated compounds, bringing attention to the use of biomass extracts known for their antioxidant content, for example, tomato waste, apple peels, grape seeds, pomace, spent coffee, etc., [135,136]. Due to their reactive nature, natural antioxidant substances present difficulties both for their extraction and their incorporation through conventional packaging production techniques due to the high temperatures used, thus requiring the use of non-conventional extraction techniques, such as extractions at high pressures and temperatures, ultrasound-assisted extraction, microwaves, pulsed electric fields, extraction with supercritical fluids and the addition of other process steps for their protection such as encapsulation, e.g. by spray drying, emulsions, liposomes, electrospun polymer fibers, polymer nanoparticles [137–139]. However, their incorporation into polymers is not easy to carry out, as they can lead to loss of properties of the packaging material in terms of reduction of mechanical properties or gas permeability. Another interesting category of natural substances with antimicrobial properties is represented by bacteriocins and enzymes [107]. Bacteriocins, such as nisin or pediocin, are peptides produced by bacteria such as lactic acid and have proven effective, even for low concentrations, against some pathogens by inhibiting the synthesis of proteins and nucleic acids in microorganisms and cell membrane permeabilization [140]. Nisin, for example, has positive charges that interact electrostatically with the negative charges of the cell membrane of the target microorganism, allowing the bacteriocin to penetrate the microorganism and cause the cytoplasmic material to escape with consequent cell death. In addition, they are suitable for applications in the food sector as they are non-toxic, tasteless, and colorless and are not particularly heat-sensitive, thus facilitating their use with conventional food film production techniques that will be described in chapter 1.6 [141]. Instead, enzymes, such as lysozyme, are able to hydrolyze the beta 1,4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine, which are the main components of peptidoglycan, a constituent of the cell wall of bacteria. Gram-positive. Unlike bacteriocins, enzymes are more sensitive to temperatures and pH variations, therefore they require their immobilization on the polymeric surfaces following the production of the polymeric films themselves, thus increasing costs. As for the synthetic components, these are, compared to natural ones, simpler and cheaper to

produce and can inhibit bacterial growth more effectively. However, the possible toxicity of these substances must always be taken into account, both for the environment and for human health, as in the case of known antioxidants and synthetic antimicrobials such as ethylenediaminetetraacetic acid (EDTA), chlorine dioxide, propyl gallate, butylated hydroxytoluene, sulfur dioxide, potassium sorbate or quaternary ammonium salts [125]. Finally, speaking of health risks, even inorganic metal nanoparticles such as gold, silver, and platinum are known to have antibacterial properties, thanks to the release of metal ions that react with oxygen causing damage to the cell walls of bacteria. However, speaking of nanomaterials, these are under scrutiny for the dubious effects they can have on health in case of ingestion or inhalation [10]. Some examples of active antimicrobial and antioxidant packaging on the market are shown in Table 1.

1.3.8 Active packaging and cold chain

Among the food preservation methods, refrigeration and freezing are some of the most widely spread and used, since the low temperatures allow to extend the shelf-life of the product by preventing microbial growth and slowing down the degradation processes. However, to properly store frozen or chilled products it is very important not to break the cold chain throughout the marketing period. If the food is exposed to temperatures higher than the optimal storage temperature during the storage and transport phases, the quality of the food may be affected. In particular, ice crystals could grow, some unwanted chemical reactions could accelerate, and the growth of microorganisms could restart. Therefore, there is great interest in finding new strategies to reduce temperature fluctuations along the cold chain. Packaging plays a fundamental role in this, since by using a packaging capable of acting as a thermal insulator, the food would be protected from any sudden changes in temperature. However, to date, only refrigerated containers are used for the transport and storage of perishable products, but the turning point would be to protect the single product from these changes, no more pallets together, also considering that, often, the temperature is not distributed evenly inside the containers. Developing a primary food packaging that helps keep food temperatures within desired limits could be a solution to this problem. However, traditional commercial packaging such as polyethylene does not provide any protection in this respect, but the concept of active packaging can, once again, be of help [142]. Indeed, one way to make packaging a thermal insulator is to use, as additives, phase change materials (PCMs) within it [143,144].

1.3.8.1 Phase Change Materials

PCMs, as the name suggests, are substances that undergo a phase transition at a specific temperature and, as a result, are capable of absorbing and releasing latent heat with a small change in temperature. An example of the operating mechanism of phase change materials is provided in Figure 16, which

shows a PCM encapsulated within a polymer matrix. As can be seen, if the external temperature reaches the melting temperature of the PCM, it undergoes a phase change and, in the transition to the liquid state, absorbs heat. Conversely, when the temperature drops below its melting temperature the PCM returns to a solid state and releases the heat previously absorbed [145].

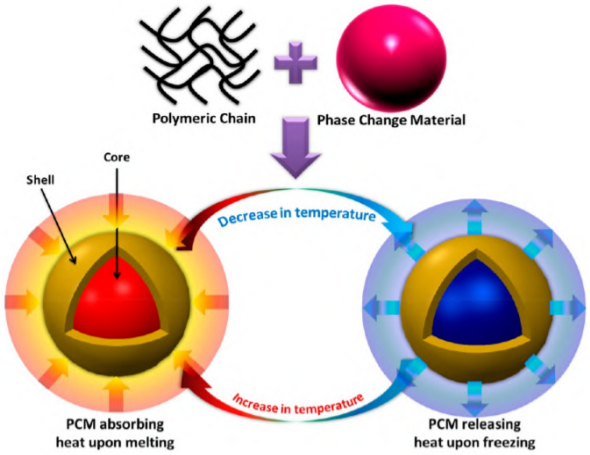


Figure 16: Schematic representation of encapsulated PCM and its heating and cooling cycle [145].

In order to be used as a phase change material, a substance must meet some physical, chemical and economic requirements [146]:

- Physical: adequate phase change temperature, complete reversible freezing/melting cycle, large enthalpy change, large specific heat capacity, high thermal conductivity and little subcooling;
- Chemicals: small volume pressure, low vapor pressure, good compatibility with other materials, chemical stability, physical stability and non-toxicity;
- Economical: low price, recyclable and abundant.

PCMs can be classified depending on their nature into:

- **Organic PCMs:** can be classified in turn into paraffinic or non-paraffinic. They have good chemical and thermal stability, are recyclable, non-corrosive and have no subcooling. Disadvantages are flammability, low thermal conductivity and a phase change enthalpy lower than that of other types of PCMs. Among organic PCMs, the most commonly used are paraffin and fatty acids. Dodecane is an example of a paraffin that can be used as a PCM in packaging. For example, Pérez-Masiá et al., used dodecane as a PCM for the production of heat-management materials for smart food packaging [143]. Dodecane is a paraffin characterized by a transition temperature of about -10 °C. Its transition temperature is low enough to make it an excellent

candidate as a PCM for protecting frozen food against temperature changes [147]. Examples of fatty acids are oleic acid and linoleic acid [148]. Linoleic acid, like dodecane, has a melting temperature of about -10 °C [148]. Therefore, it could be used for the production of active packaging with thermal insulation properties for frozen food. Oleic acid, on the other hand, has a transition temperature of about 4-8 °C, therefore it would be more suitable for the protection of refrigerated foods [149].

- **Inorganic PCMs:** these include hydrates salts, salts and metals. The advantage in this case is given by the higher enthalpy of phase change, but on the other hand they are subject to corrosion, subcooling, phase segregation and do not present a good thermal stability. For salts and hydrates salts, mixtures of several compounds are often used. An example of a salt mixture that can be used as a PCM is the sodium nitrate ($NaNO_3$) and potassium nitrate (KNO_3) mixture [150].
- **Eutectic:** is a mixture of substances whose melting point is lower than that of the individual substances that comprise it. Mixtures can be organic-organic, inorganic-inorganic, or inorganic-organic, and there are many that are suitable for use as PCMs. An example of a eutectic mixture that can be used as a PCM is the n-tetracosane and n-octadecane mixture [151].

To date, several studies related to insulated boxes and refrigerated equipment with PCM, for example ice cream tubs, can be found in the literature [152,153]. However, little information exists in the literature about the incorporation of encapsulated PCM structures into polymeric matrices for food packaging purposes. Among these few works, Pérez-Masiá et al., developed a material based on zein loaded with dodecane by electrospinning to produce a material intended for frozen food packaging [143]. The work of Chalco-Sandoval et al., was instead aimed at producing food packaging with thermal insulation properties for refrigerated food using Rubitherm RT5, a technical grade paraffin wax, loaded into polystyrene matrix via electrospinning [144]. A study similar to that of Chalco-Sandoval et al., is the one conducted by Hoang et al., in which the authors have used Rubitherm RT5 loaded in polycaprolactone electrospun fibers [152].

As mentioned there are few works in the literature dedicated to this topic, but an aspect highlighted by these works is the need to encapsulate these phase change materials in order to be able to incorporate them into the polymeric supports in order both to protect them from the external environment and to protect the environment and, in particular, the food product, from their eventual release, which in this case, unlike migratory active packaging, must not occur, both for reasons of possible toxicity in the case of use of inorganic PCM and to prevent the loss of these compounds which would lead to the loss of functionality of the packaging itself. Furthermore, among the encapsulation techniques, electrospinning seems to offer a good technological solution as it is a technique that allows the encapsulation and production of polymeric films in a single step. At the

end of this extended chapter on active packaging, Table 1 shows some of the systems described currently on the market and the related food application.

Table 1: Commercially available food active packaging. Adapted from [107].

Commercial Name	Principle	Form	Application
Activ-Film™ (www.csptechnologies.com)	Moisture absorber	Low-density polyethylene film	Fruit and vegetables
Tenderpac® (www.sealpacinternational.com)	Moisture absorber	Polyethylene terephthalate tray	Meat products
Celox™ (www.grace.com)	Oxygen scavenger	Cans sealants and closure coatings	Beverages
ZERO$_2$ (www.ipl-plastics.com)	Oxygen scavenger	Multilayer films	Fresh products
PEAKfresh™ (www.peakfresh.com)	Ethylene scavenger	Film impregnated with a natural mineral	Fruit and vegetables
BIOPAC (www.biopac.com.au)	Ethylene scavenger	Sachet with potassium permanganate	Fresh products
ATCO® (www.emcotechnologies.co.uk)	Carbon dioxide absorber	Film or bags	Fresh products
McAirlaid's CO2Pad (www.mcairlaids.net)	Carbon dioxide emitter	Cellulose-based pads	Fish, meat and fruit products
FreshPax® (www.multisorb.com)	Carbon dioxide emitter	Films based on food grade materials	Processed and pre-cooked food
Biomaster® (www.biomasterprotected.com)	Antimicrobial	Cool bags	Chilled and frozen products
Food-touch® (www.microbeguard.com)	Antimicrobial	Paper in various forms	All food products
ATOX (www.artibal.com)	Antioxidant	Film coating with oregano essential oil	Cereal products
Pure Temp (www.puretemp.com)	Phase Change Materials	Palm, coconut and soybean oil	Frozen food, cold storage
Green Box (www.greenbox.it)	Phase Change Materials	Vegetable oil-based	Perishable products

1.3.9 Intelligent packaging

The function of intelligent packaging, as mentioned, is not to intervene directly in the quality of the products and the extension of the shelf life but rather to provide information on the performance of the packaging system. These systems have the purpose of guaranteeing food safety, guaranteeing traceability throughout the food chain, facilitating communication, and supporting a decision-making process, which concerns both the choice of the product by the consumer and the decisions that must be taken by the manufacturer to improve the logistical aspects along the distribution chain. For example, instead of relying on the 'use preferably by' label to indicate product freshness, smart packaging can provide real-time visual feedback on the status of the content, taking into account potential chemical, microbial and physiological changes in the product itself. The information that is collected and communicated by smart packaging is that which can be easily traced back to the quality status of the food, such as temperature variations in the environment outside the packaging, or the presence of specific metabolites inside the packaging [154]. Furthermore, these systems offer a powerful means of monitoring and risk reduction, applicable in HACCP (Hazard Analysis and Critical Control Points) system procedures [155].

There are different types of intelligent packaging that can be classified, depending on their function, in indicators, sensors and data carriers [156] and, unlike active packaging, these can be positioned both inside or outside the primary packaging, but also on secondary and tertiary ones if their purpose is linked to logistics [104].

1.3.9.1 Indicators

The indicators provide qualitative or semi-quantitative data regarding the quality status of a product through visible and irreversible changes in the intelligent system itself, generally through a change in the color of the indicator. Depending on the final application and the materials with which they are made, the indicators can be positioned both inside and outside a primary packaging [157]. In any case, their principle of operation is based on the control and detection of a variable, such as the presence or absence of target substances, changes in pH, the presence of volatile compounds produced by food degradation reactions, or keeping track of temporal data relating to storage conditions, such as temperature. Among the indicators, the most known and investigated ones currently are:

- **Freshness Indicators:** they are systems that track the deterioration and loss of freshness of packaged products and are widely used for quality control of foods such as fruit, vegetables, and meat and fish products along the distribution chain [158]. The loss of freshness is associated with the presence, in unwanted quantities, of compounds such as organic acids, glucose, carbon dioxide, ethanol, amines, sulfur derivatives (for meat) and volatile nitrogen compounds (for fish).

Generally, these systems consist of multiple polymeric layers impregnated with dyes sensitive to pH variations induced by the degradation processes of fresh foods, designed so that migration of these into the food cannot occur, an example is shown in Figure 17, while some of the systems available on the market are shown in Table 2. The most used dyes are bromocresol purple, bromocresol green, bromophenol blue, methyl red, and cresol red [107], but also natural extracts [159].

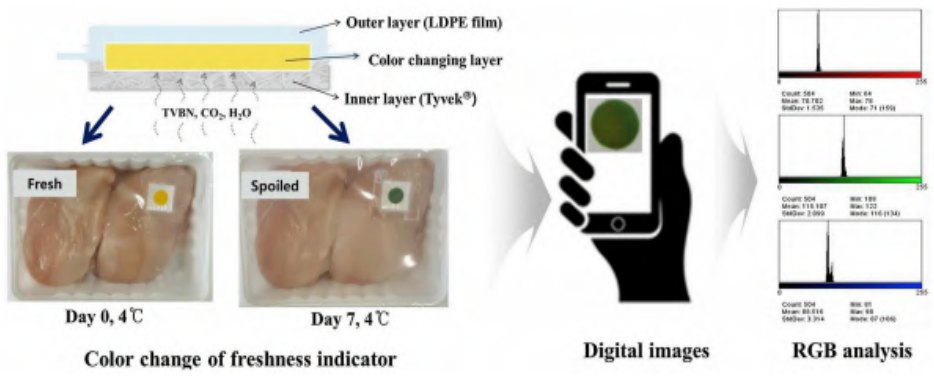


Figure 17: Polymeric multilayer freshness indicator [157].

Table 2: Commercially available freshness indicators.

Commercial Name	Application	Principle
Raflatac (www.upmraflatac.com)	Poultry	Nanolayer of silver that reacts with hydrogen sulfide, a breakdown of cysteine
RipeSense® (www.ripesense.co.nz)	Fruit	It detects ethylene produced in the ripening process
Food fresh™ (www.vanprob.com)	Meat	Self-adhesive label indicators that can be set to time out within a given 'consume within' time frame

- **Gas Indicators:** the composition of the gas inside a package can change due to the interactions of food with the surrounding environment [156]. The gas indicators, placed in direct contact with the headspace of the packaging, are a useful method to control the possible production of unwanted gases produced during the decomposition of the food or to detect any leaks in products

packaged in a modified atmosphere (MAP) [160]. The most common gas indicators are those for the detection of oxygen and carbon dioxide, as also shown in Table 3 dedicated to the systems on the market. This type of indicator is based on changes in color as a result of reducing oxide reactions between a dye such as methylene blue and glucose, placed in an alkaline solution [161,162]. In the case of carbon dioxide indicators, these colorimeter systems respond to pH changes and those based on the use of anthocyanins are receiving considerable attention [163,164].

Table 3: Commercially available integrity indicators.

Commercial Name	Application	Principle
Ageless Eye® (www.mgc.co.jp)	Meat	Sachets contain an oxygen indicator tablet
Tell-Tab™ (www.impakcorporation.com)	All products	In-package label which indicates oxygen presence
O_2Sense™ (www.evigence.com)	Fresh products	Eye-readable indicator to detect leakages in modified atmosphere packaging MAP
Novas Insignia Technologies (www.insigniatechnologies.com)	Products packed in a modified atmosphere	It shows when packaging has been damaged
Shelf-Life Guard (www.upm.com)	Meat	Detect air in modified atmosphere packaging

- **Time-Temperature Indicators (TTI):** due to their simplicity, efficiency, and low cost, these indicators are widely used to monitor and communicate information on the quality of food products. Unwanted temperature fluctuations can in fact lead to the production of huge quantities of food products, especially those for which compliance with the cold chain is required [165]. TTIs can respond to a temperature change only if it exceeds a set limit, and then they are defined as partial history indicators, or they can track temperature throughout the supply chain by assuming the definition of a full history indicator [166]. Some of the indicators available on the market are shown in Table 4 and can base their operation on chemical reactions of polymerization, mechanical modifications in terms of deformation, changes in color such as the example shown

in Figure 18, or, for the more expensive version, they are sensitive to the biological activity of enzymes and microorganisms [167].

Table 4: Examples of commercially available time-temperature indicators.

Commercial Name	Application	Principle
3M™ MonitorMark® (www.3m.com)	Backery, beverage, meat	Self-adhesive pad containing a blue dyed fatty acid ester for secondary packaging to monitor temperature exposure.
Fresh-Check® Temperature Intelligence™ (www.fresh-check.com)	All fresh products	Self-adhesive device based on solid state polymerization reaction resulting in highly colored polymer.
OnVu™ (www.packworld.com)	Meat, fish and dairy products	Composed of a photochromic ink based on benzylpyridines activated by UV light
TempDot® (www.deltatrak.com)	Seafood and meat	Labels that can be shipped and stored under any temperature
Freshtag/Check point ® (www.vitsab.com)	Meat, fish and dairy products	Controlled lipolytic hydrolysis of substrate by enzymes triggers pH reduction and color change from green to red
Traceo® (www.cryolog.com)	Chilled food products	Transparent adhesive label in which selected strains of lactic acid bacteria are trapped
eO® (www.cryolog.com)	Cold chain	Small gel pad in which the color change represents a pH change due to microbial growth of lactic acid bacteria

Figure 18: CheckPoint® time-temperature indicator marketed by Vistab [168].

1.3.9.2 Sensors

Sensors used as intelligent packaging systems are devices designed to respond with a quantifiable signal to changes in properties such as temperature, pH, humidity, development of gaseous compounds, etc. that can occur inside a food packaging. Sensors are generally composed of a receptor, or sampling area, which represents the part sensitive to chemical or biological variations; a transducer, which detects and converts the receptor signal into an output signal. Finally, the signal processing takes place thanks to an electronic component and the display of the signal on a display unit [106,166]. To date, these devices are extensively investigated but their commercialization is still far behind, as can be seen from the scarcity of commercial products reported in Table 5, due to problems related to detection sensitivity, selectivity for the target variable, need for miniaturization, and cost reduction, as well as the need to be able to work without secondary portable tools [169]. A greater success of these devices is expected in the near future thanks to the use of nanotechnologies for the incorporation of sensors in the flexible form directly into the packaging material and even for the realization of edible sensors to be placed in direct contact with the food. For now, these solutions are still under study, in particular, due to the debate and the lack of legislation regarding compounds at the nanoscale [170]. However, the sensor category is very extensive and includes both chemical and biosensors.

- **Chemical Sensors:** they have the purpose of detecting and indicating the presence of gaseous or volatile compounds such as carbon dioxide, oxygen, volatile amines, etc., associated with the deterioration of food. The purpose is the same as for gas indicators, but in this case, the transducer signal is of the electrochemical or optical type. In the first case, the transducer is an electrode generally made with inert metals or metal oxides, but more and more interest is arousing biodegradable electrodes based on activated carbon to reduce costs and environmental impact [169], and the use of nanomaterials carbon-based, in particular nanofibers [155]. Systems of this type have been studied, for example, for the detection of bisphenol-A released from plastic packaging [171], or for the detection of antibiotics in milk [172]. Perhaps the best-known chemical sensor is the e-nose, a complex device for detecting odors developed inside the packaging due to the production of volatile compounds linked to food spoilage, but it is also used as a monitoring system in processes such as fermentation [173]. In the case of sensors with an optical signal, this signal, which can be colorimetric, fluorescence, or chemiluminescence, can be detected with the naked eye or by photo-detectors. The most used materials are optical fibers, dyes such as platinum-porphyrins or ruthenium-porphyrins in association with polymers [174], or silicon-based transducers [175]. For now, these devices still have problems related to signal interference with the external environment and the high operating costs associated with the need to use lasers and infrared detectors for reading.

- **Biosensors:** these devices are defined as "biosensors" as the receptor is made up of biological material (DNA, enzymes, antigens, hormones, etc.) which must be immobilized in the receptor by means of electrodeposition, for example. The biosensors are therefore suitable for the detection of allergens, analytes, metabolites, and pathogens [106,166], and can be both electrochemical and optical depending on the transducer. A successful example of an electrochemical biosensor is represented, in the biomedical field, by the device for detecting an electrode in the blood, consisting of an enzyme-based electrode [176]. In the food sector, an example of an electrochemical biosensor is a device made of nylon for the detection of glucose in drinks or meat, as an index of deterioration [157]. Optical biosensors, on the other hand, are mainly dedicated to the detection of pathogens, such as *Escherichia Coli*, *Salmonella*, *Listeria*, and *Campylobacte*r and are based on antibody and antigen reactions that give rise to color changes of the biosensor [166]. The main limitation of these devices remains the immobilization of these biological substances on the sensor and its integration into the packaging material so that food contamination cannot occur.

Table 5: Commercially available sensors.

Commercial Name	Application	Principle
O2xyDot® (www.oxysense.com)	All products	Optical sensor placed in transparent or semi-transparent packages to measure oxygen
Flex Alert (www.flex-alert.com)	Coffee beans, dried nuts, seeds, wine barrels and fresh fruit	Flexible biosensor to detect toxins in packaged foods throughout the supply chain. It has been specifically developed against *Escherichia coli*, *Listeria* spp., *Salmonella* spp., and aflatoxins

1.3.9.3 Data Carriers

Data carriers represent a particular class of intelligent systems, developed not so much to provide information on the quality of food, but rather to facilitate logistics and efficiently guarantee the traceability of products. These devices are mainly applied on tertiary packaging to be easily readable, but the way to apply them directly to primary packaging is also being studied in order to be able to exploit them also for the detection of product quality. The category of data carriers includes:

- **Barcodes:** they can be described as an archiving database that can contain a multitude of particular data such as the article number, the identification number of the manufacturer, the date of packaging, the weight of the package, and nutritional information. The stored data can be read through a device whose function is based on optical phenomena. The first barcode was marketed as the UPC (Universal Product Code) and is now widely used in stores for effective stock

reordering and inventory control. This is a one-dimensional code based on twelve numerical digits and on the arrangement of alternating black and white bars, spaced at regular width and thickness. More modern barcodes are instead made up of other shapes and symbols or, even newer, QR-codes (Quick Response) that use two-dimensional geometric patterns [166].

- **Radio frequency identifiers (RFID):** they are part of those devices that have the purpose of following the movement of food through specific stages of production, transformation and distribution. RFIDs, the main components of which are shown in Figure 19, can be classified into three types: active, passive, and semi-passive depending on the type of power supply and the signal transmission distance [177]. They are systems based on radio waves that, in wireless mode, can collect data in real-time and therefore take care of the traceability of the product, providing a guarantee of the integrity of the package and the safety of its contents throughout the supply chain. These devices, although more expensive, have several advantages for product identification compared to other systems such as barcodes. An example is given by the fact that RFIDs do not require visual contact from an external device and therefore can be placed inside boxes and containers, while still allowing them to be read even from a distance. Some examples of commercially available RFID tags are shown in Table 6.

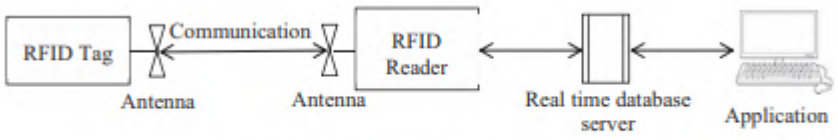


Figure 19: Operation diagram of an RFID system applicable to food packaging [168].

Table 6: Commercially available RFID technology.

Commercial Name	Application	Principle
Easy2log® (www.environmental-expert.com)	Seafood, meat and poultry, milk-based products, frozen food	Semi-passive tag that allows monitoring temperature-sensitive products during transportation and storage
CS8304 (www.convergence.com.hk)	Cold chain	The tag provides 10.000 samples of logging memory for saving of temperature data. LED light indicates temperature violations
TempTRIP (www.temptrip.com)	Cold chain	This temperature tag uses ultra-high frequency to communicate wirelessly to readers that send the results directly to Internet Web page

1.4 Regulatory Aspects

Before the development of the concept of active packaging, European legislation concerning materials and objects intended to come into contact with food was very clear and required the packaging to be completely inert.

In particular, the principle underlying Regulation 1935/2004/EC was that "materials or objects intended to come into contact, directly or indirectly, with food products, should be sufficiently inert, to exclude the transfer of substances to products food in quantities such as to endanger human health or to lead to an unacceptable change in the composition of food products or a deterioration of their organoleptic characteristics" [178].

The problem that arises with the development of active packaging is that these, by definition, are designed to interact with the foods contained. For this reason, active and intelligent packaging, in addition to being subject to some of the regulations of traditional plastic packaging, must follow additional regulations.

For active packaging, in particular, regulation (EC) no. 450/2009 of 29 May 2009, defines that active substances can be incorporated directly into the primary packaging or externally, in a separate container, in any case, their presence must be adequately reported. For example, if the active components are not incorporated in the packaging but placed in an external container, the latter must be reported as inedible. Furthermore, in addition to the presence of an active substance, the respective released quantity must also be reported and distinguish between active substances with additives for migratory systems, for which therefore the controlled release on the food is desired, and non-migratory systems, for which there must be no release, or rather it must be subject to regulated limits [105]. As mentioned before, active packaging poses new challenges in assessing its safety, precisely because of the substances that can be released. This is why the regulation (EC) n. 1935/2004 of 27 October 2004 (Article 7), states that active packaging must not alter the composition and organoleptic properties of food products and must not be used to cover and mask the deterioration of food. The use of substances that cause a change in color, aimed at making a deteriorated product look fresh, is also not allowed, in order not to mislead and protect the consumer. For these reasons, the use of aldehydes and amines to cover deterioration is prohibited.

In subsequent regulations that rectify that of 2004, i.e., EU Regulation no. 10/2011, EU Regulation 2016/1416, and the Regulation (EU) 2020/1245 of the European Commission concerning plastic materials and objects intended to come into contact with food products, in fact, the methods for the evaluation of overall (OML) and specific (SML) migration limits for materials intended to come into contact with food [179–181]. In particular, for the demonstration of conformity of a material, the food simulants and the conditions in which to carry out the tests are defined.

The approved food simulants are as follows:

- Simulant A: Ethanol 10% (v/v), for hydrophilic foods;
- Simulant B: Acetic acid 3% (w/v), for foods with a pH lower than 4.5;
- Simulant C: Ethanol 20% (v/v), for food products with alcoholic and lipophilic content;
- Simulant D1: Ethanol 50% (v/v), for lipophilic products;
- Simulant D2: Vegetable oil or Ethanol 95% (v/v) and isooctane, for products containing free fats;
- Simulant E: poly (2,6-diphenyl-p-phenylene oxide), for dry products.

Depending on the methods and storage times envisaged for the food product, the contact conditions (between material and simulant) are also listed, in terms of time and temperature, considered the most severe to be tested.
All voluntarily added substances must be evaluated and approved by EFSA, the European Food Safety Authority, and by the FDA at an international level. In addition to testing substances, their possible combinations are also analyzed. In particular, their safety is evaluated, that is, they must not be toxic, mutagenic or carcinogenic, with particular reference to nanomaterials. Risk management is also taken into account and must be minimized.

1.5 Methods for manufacturing polymeric films

Considering the manufacturing techniques of flexible films for active packaging, it must be considered that in order to make the use of biopolymers attractive to replace fossil polymers, the possibility of processing them with conventional techniques such as extrusion, molding, calendering and casting remains fundamental. Speaking of active packaging, it should be considered that the active compounds can be incorporated directly into the polymer matrix and then processed together with the polymer with traditional techniques if they are not thermolabile, or they need to be treated with more innovative techniques, less present on an industrial scale, which work in mild conditions. The second option is to add active compounds at a later stage to the manufacture of the polymer support by, for example, electrostatic immobilization, or plasma treatment, or impregnation, for instance with supercritical fluids. In this chapter, both some of the traditional techniques for the production of polymeric films and some of the absolutely non-innovative techniques but certainly new for application in the field of packaging will be discussed, with particular reference to the solvent casting and electrospinning techniques that have had a leading role in this Ph.D. work.

1.5.1 Extrusion Methods

In the traditional extrusion process, a starting polymer (powder, granules, flakes, pellets) is fed into a screw extruder which heats and melts the polymer. The molten polymer is then forced into a mold

and the resulting polymer flat sheet is collected in a rotating drum which cools the films below its melting point. Subsequently, a series of rollers complete the cooling effect. The thickness of the film obtained with this technique is generally between 0.01 and 0.1 mm [182]. A variant is the blow extrusion technique which consists in producing a resin by dissolving the polymer in an appropriate solvent. The mixture is heated to a certain temperature for homogenization, then poured into a water bath containing ice to form a slurry which is then passed through a single screw extruder. Finally, the polymer is extruded onto a balloon using the blow head to form the film [183]. A more advanced version is coextrusion, which consists in coupling two or more extruders that feed different resins to a single mold head to simultaneously extrude different polymers that melt at the point of formation of the film into a single tape. This process allows the production of a single layer which has, for example, barrier properties not possessed by any of the individual components. Today, seven- or nine-layer films are produced for food packaging applications [6].

1.5.2 Molding Methods

The compression molding process is widely used to produce objects from thermosetting materials such as resins. The mold is made up of two perfectly overlapping parts, whose space gives shape to the printed object. During the compression molding process, heat and pressure are applied simultaneously when the mold containing the polymer is closed so that the material becomes plastic to flow and fill the mold. The molds are held together until all the resin has hardened and this can take from a few seconds to several minutes, then it is opened and the object is removed [182]. Another version of molding is injection molding, a high-speed technique used for the production of thermoplastic and thermosetting polymers. The polymer in the form of powder or liquid resin is fed into a hopper and forced into a horizontal cylinder, where it is softened thanks to electric heating plates. Pressure is applied to the molten material via a hydraulic piston in a mold. Inside the hot cylinder, a device called a "torpedo" helps to evenly accelerate the plastic material around the wall ensuring even heat distribution. The molten material is then injected through a nozzle into the mold cavity. The polymer after being solidified with the desired shape can be extracted. This cycle repeats several times and the sequence usually only takes about 10-30 seconds, so the method is quite suitable for large-scale production but the price of steel molds is not to be underestimated [6,183]. More recent development of injection molding is reaction injection molding. In this process, two resins are injected together into the mold. Just before they enter the mold, a chemical reaction takes place between the two and this process requires no additional heat for molding [184].

1.5.3 Calendering

In this process, the plastic compound is passed between a series of heated rollers, from two to five rollers [6]. The sheet exiting the rollers is cooled by passing through other cold rollers. The sheets are then collected by wrapping them in rolls. The thickness is controlled by the speed of the finishing rollers [182]. The surface finish of the material is determined by the last roll and can be glossy, matte, or rough. Calendering usually produces films with better thickness uniformity than that obtained by extrusion [185].

1.5.4 Solvent Casting

Solvent casting is a rather old method widely used in the field of packaging films due to its ease of production and cost-effectiveness [186]. Solvent casting has three main steps, namely polymer solubilization, solution casting and drying [183]. First, the polymer is dissolved in a suitable solvent to obtain a viscous solution, which is then poured onto a flat non-adhesive surface. Once the solution is homogeneously distributed, the solvent is allowed to evaporate and the dry films can then be removed from the flat surface. To avoid the formation of weak conglomerates in the polymer solution, a suitable molecular weight of the polymer is necessary (higher is better). To facilitate the pouring of the solution, the solvent must be volatile enough to evaporate at a reasonable rate at room temperature or at mild temperatures. However, the solvent must not evaporate too quickly to avoid the formation of bubbles or semi-crystalline precipitates. Rapid volatilization also causes the film to cool, which could then cause cracking or condensation of water from the atmosphere [182]. On a small scale, the polymer film can be prepared in the laboratory simply by spreading the polymer solution on a smooth, leveled surface with only the limit of the surface extension that can be produced. On a large scale, the process is slightly different (Figure 20): the polymer solution is fed continuously through a hopper on a stainless-steel belt over which a flow of air with an adequate temperature is passed. The speed of the belt, which depends on the rollers to which it is connected, must be extremely constant and there must be no vibrations of any kind to avoid the formation of defects. The process ends, once the solvent has finished evaporating, by removing the film with the aid of a blade and collected in a coil [182]. The main parameters to control are the composition of the solution, i.e., the compatibility between the polymer and the solvent and also the compatibility of the solvent with the active compounds in the case of manufacturing an active packaging. Furthermore, the viscosity of the polymer solution is of fundamental importance as it must be easy to spread on the support. The evaporation rate and the evaporation temperature of the solvent for the reasons already mentioned above. It must be ensured that there is no air trapped in the solution which during evaporation would lead to the formation of bubbles. Additionally, some polymers may be too tacky to remove from the substrate once dry, so a plasticizing agent is often used to facilitate the dry

film removal operation [187]. In addition, the use of a plasticizing agent also allows to improve the mechanical properties in the case of biopolymers, but the amount of plasticizer must be well weighted to avoid the opposite effect. Through this technique it is possible to obtain isotropic transparent films, with good optical properties and with a wide range of thicknesses, from a few microns to a few millimeters.

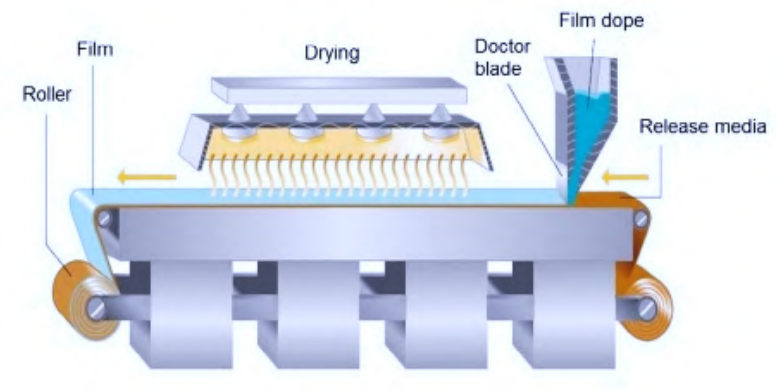


Figure 20: Equipment for industrial film production by solvent casting [188].

Through this technique, it is also possible to obtain transparent but porous films, through the use of a porogen agent dissolved in solution, which after drying solidifies together with the polymer and is washed away by a solvent that redissolves it, leaving pores of controllable dimensions. Typical non-toxic and inexpensive porogens are salts, such as sodium chloride or calcium carbonate, which can be removed by simply washing the film in water. This variant of solvent casting is called particulate leaching [189].

1.5.5 Spinning Methods

There are several spinning techniques, mainly distinguished between wet spinning, dry spinning and melt spinning. In the wet spinning technique, a motorized syringe pump is used to push the viscous polymer solution into a coagulation bath that contains an anti-solvent whereby the polymer solution precipitates and the formed fibers can be washed, finished, and finally rolled up [190]. In the dry spinning method, a polymer fiber is formed using hot air to dry the fibers, which are extruded from the syringe. In practice, the polymer solution is pushed downwards by a syringe into a vertical cylindrical tube and at the same time hot air is blown from the bottom upwards. When hot air is blown into the chamber, the flowing polymer solution becomes a solid wire, which can be rolled into a coil [191]. Instead, in the melt spinning, solid polymers are heated to melt in a grid. The melt

solution is filtered by applying pump and it is forced down through the spinneret. As soon as the fine threads come out through the spinneret, a stream of cold air is passed on the threads so as to solidify the fibers. The solid form of threads is passed between two oppositely rotating drums followed with finish applicator to get uniform diameter of the fibers and finally the fibers are wound up in a coil [192].

1.5.5.1 Electrospinning

Electrospinning is a relatively simple technique that allows nanofibers to be produced from a wide variety of polymers. Using this technique, in fact, fibers with diameters ranging from 2 nm to several micrometers can be produced using polymeric solutions of natural and synthetic polymers [193]. This interesting technique has been studied for years in the biomedical field for the production of polymeric scaffolds, and is already used on an industrial scale for the production of filter membranes [194], while it has only recently attracted attention as a film production technique for food packaging [195,196] A schematic representation of the working principle of electrospinning, which is based on the use of a high-voltage electric field, is shown in Figure 21.

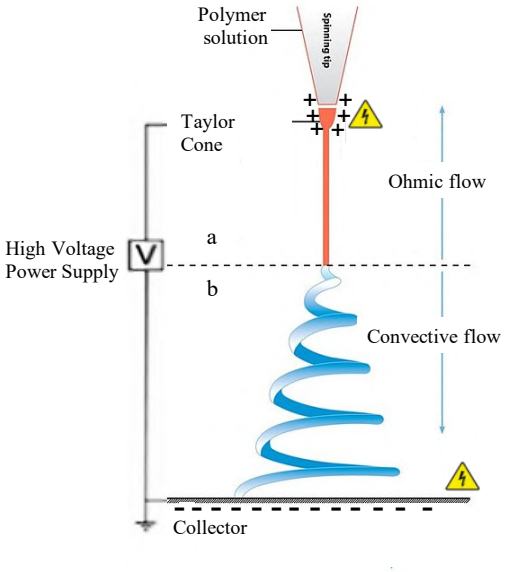


Figure 21: Schematic illustration of a conventional electrospinning process.

The main components of the equipment are a syringe into which the polymer is loaded, which may be melted or dissolved in a solvent, a high voltage power supply and a collector. The syringe is then usually connected to a digitally controlled pump through which the feed rate can be adjusted. The

polymer solution is pumped into a millimeter-sized metal needle that is connected to the high-voltage power supply. When a certain critical voltage (5-35 kV) is reached, the electrical force overcomes the surface tension of the droplet at the syringe tip and the droplet of polymer solution distorts and forms a Taylor cone and is then ejected as a charged polymer jet. This jet is then stretched partially in the form of an expanding helix and is accelerated by the electric fields toward the grounded and oppositely charged collector. As the electrospun jet travels through the electric field, the solvent evaporates completely and ultra-thin polymer fibers are deposited on the collector forming non-woven mats [112]. Electrospinning is very similar to electrospraying, a process whereby liquid is atomized by electrical forces. The electrospraying setup, in fact, is almost identical to the electrospinning setup. The result obtained by using the two techniques, however, is quite different. In electrospraying, in fact, solid particles are deposited on the collector, while in electrospinning a non-woven mat is formed. The main difference between the two techniques lies in the concentration of the polymer solution. When the concentration of the solution is high, the jet from the Taylor cone is stabilized and the mechanism that occurs is as described above. If the concentration is low, however, the jet is destabilized and fine droplets are formed due to Rayleigh-Plateau instability which causes jet atomization. These highly charged droplets self-dissipate in space and, consequently, do not undergo agglomeration and coagulation phenomena. Finally, as in the electrospinning technique, the solvent evaporates. As a result, the droplets contract and solidify before depositing on the collector. The Taylor cone, therefore, plays a fundamental role in the electrospinning process, since the success of the process itself depends on its stability. In fact, in the literature there are many works dedicated to the modeling and fluid dynamics simulation of the Taylor cone [197–199].

The process is also influenced by many other parameters including:

- **Polymer solution properties**: polymer molecular weight, polymer concentration, solution viscosity, conductivity, surface tension;
- **Processing conditions**: flow rate, applied tension, distance between the tip and the collector, type of collector;
- **Environmental conditions**: temperature and humidity.

These parameters significantly influence the morphology and properties of electrospun fibers, so they must be carefully studied and/or monitored to achieve the desired result.
In addition, the electrospinning setup can be modified in various ways (Figure 22) to adjust the collection, geometry, and orientation of the fibers, enabling the fabrication of nanofibers for a variety of applications. By changing the type of collector, samples of different shapes can be obtained, for example films or cylinders of different sizes. Some examples of collectors are: rotating mandrel, rotational collector, moving spinneret, grounded drum collector, flat rectangular collector, grounded

rotating drum, aluminum foil, aluminum foil-covered metallic collector and copper grid. The type of needle is also very important in the electrospinning process. For example, a simple needle can be used, or, as in coaxial electrospinning, two concentrically arranged needles [112].

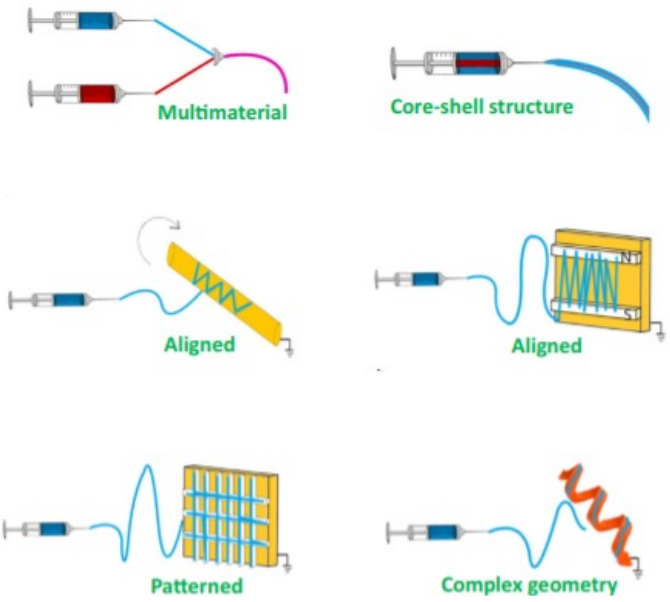


Figure 22: Schematics of electrospinning designs. Adapted from [112].

The material obtained is a porous film, consisting of more or less intertwined fibers depending on the support used for the process and, as such, it is not able to offer protection against the permeability of gas. Therefore, the material is not suitable to be used for foods that instead need to be protected from oxygen or water vapor, but offers an unbeatable structure as regards the production of migratory active packaging, such as antimicrobial and antioxidant ones, due to the high surface exposed by the fibers. However, electrospinning is suitable for the production of active patches to be inserted in the primary packaging or, in the primary packaging, as an active layer in a multilayer film. In fact, the electrospinning technique also allows to give additional functionality to the polymer by incorporating active compounds into the starting solution [112,144]. By doing this, it is possible to encapsulate the additives in the polymer matrix. In fact, the addition of these substances directly in the solution, results in a material composed of ultrathin hybrid fibers in which the active compounds remain packaged as liquid or solid nanoparticle nanodroplets. Furthermore, the electrospinning process takes place at room temperature, so this technique is more suitable for encapsulating thermally labile active agents than fibers made with the conventional melt spinning process. Compared to other techniques, it also has the great advantage of allowing encapsulation of the active compounds simultaneously

with sample production. Other advantages of electrospinning are the possibility of using different biopolymers, which is very important from the perspective of environmental sustainability, and its low cost [196].

1.6 Requirements and properties of active food packaging

So far, the main advantages and limitations of biopolymers as packaging materials compared to plastics have been brought to light. The main functions performed by packaging, namely containment, protection, convenience and communication, were mentioned and the additional "accessory functions" it can perform if designed as active packaging were discussed. At the end of this extensive section dedicated to the state of the art of food packaging, the main requirements desired in a material to be used as food packaging are now reported. In general, these properties depend on both the nature of the polymer and the manufacturing method of the material and the environmental conditions in which the material is stored [200]. However, it is known that plastic materials satisfy the requirements listed below very well, while biopolymeric materials tend to be devoid of one or more properties. The main properties required of a food packaging material are:

- **Mechanical Properties:** among the physical properties required of the packaging material, the mechanical ones are among the most important. These properties can relate to creep resistance, mechanical resistance, tear resistance, puncture resistance, torsion resistance, or even vibration resistance. Among these, the mechanical strength is particularly suitable for expressing the behavior of a packaging material subjected to external stresses, and in the case of polymeric films, the evaluation of the tensile strength carried out according to defined standards (UNI EN ISO 527) [201], is more suitable than, for example, the evaluation of compressive strength, which is more ideal for three-dimensional objects such as cans. From these tests it is possible to obtain, through stress-strain diagrams, quantitative information useful for the comparison between different polymeric materials. This information, which constitutes the technical specifications of the material, is expressed in terms of elasticity, that is the ability of a material to deform without breaking; tensile strength; fragility, i.e., absence of plastic deformation; ductility, or the degree of elongation or deformation reached by the material at the time of rupture; toughness and resilience, i.e., the energy that can be absorbed by the material before breaking. Most biopolymers generally have poor mechanical properties compared to plastics and this represents one of the limiting factors for their use on an industrial scale. However, as reported in the literature, it is possible to improve these properties through various methods.

  A first method is to use plasticizing agents to increase elasticity. A plasticizer, such as polyols, fatty acids, polyethylene glycol, and glycerol, acts on the polymer chain by increasing the free

volume and making it more mobile, also lowering the glass transition temperature [202]. The main difficulty of this method lies in identifying the right plasticizer concentration to use because above a certain limit there is the risk of obtaining the opposite effect, i.e., an increase in fragility [203].

A second method is cross-linking, to increase its tensile strength, thanks to the formation of inter- and intra-molecular covalent bonds between the polymer chains. This technique, particularly useful in the case of proteins thanks to the presence of functional groups, can be carried out through the use of chemical agents (formaldehyde, glutaraldehyde, phenolic compounds), treatments with radiation (UV or gamma rays), or through the use of enzymes (transglutaminase) [204–206]. Or it is possible to improve these properties through the manufacture of composite films, multilayers, or polymeric blends that allow the exploitation of the advantages of different polymers to create one with better performance than single ones [207,208].

- **Gas Barrier Properties:** gas barrier properties mean the ability of a material not to permeate gases, such as oxygen, water vapor, and carbon dioxide, all gases which, as already mentioned, negatively affect the quality of food but also flavorings. The permeability of a material, and in particular the phenomena that describe it, i.e., the adsorption, diffusion, and solubility of gases, depending on various factors, including the density of the polymeric material, the degree of crystallinity, the affinity between the permeating gas and the polymer, the relative humidity of the environment and the temperature obviously [209,210]. Finally, the methods of production of the material, for example, the presence or absence of plasticizers, added to improve the mechanical performance, increases the permeability as they make the polymer more elastic, thus favoring diffusive phenomena. Even in the case of gas barrier properties, biopolymers are for the most part less performing than plastics, although synthetic polymers such as LDPE and HDPE offer a low oxygen barrier, sometimes lower than that of biopolymers [211]. Among the methods reported in the literature to improve the impermeability of a film, the introduction into the polymeric matrix of solid particles or nanofillers seems to offer a good solution, as they decrease the diffusion of the gas making it more tortuous, but this involves reductions in mechanical performance as discontinuities are introduced into the material. Another technique is the orientation of the polymer macromolecules, for example by stretching, as their alignment leads to the reduction of intermolecular spaces with a consequent decrease in diffusion. Even in the case of permeability, the production of multilayer or laminated films can improve the properties of the resulting material or the deposition of waterproof coatings using a plasma technique [212–215].
- **Wetting Properties:** the wettability of a material represents a physical surface property that depends on the surface tension of the liquid (which could be the food itself or part of it, or inks used for labeling) and of materials [216]. This property gives information on the hydrophilicity

or hydrophobicity characteristics of a material and, therefore, it is essential to know in order to decide what types of foods the material may be suitable for, being an indication of its resistance to water or oil. Among the methods for evaluating the wettability of a polymeric film, the best known is certainly the static method of the sessile drop, but there are other methods, for example, the Wilhelmy dynamic method [216,217]. Plastics generally have low surface tensions, therefore low wettability and as such can cause droplet formation problems inside the packaging of high humidity products as well as problems in choosing suitable inks. In the case of biopolymers, the wettability properties are particularly important because they also determine the adhesive properties between polymers and therefore the possibility of producing multilayer films. The treatments to increase the surface tension and therefore the wettability of material are corona treatment, plasma treatments, or the application of coatings, all of these methods have the ability to make the polymeric surface more polar and therefore more wettable [218,219].

- **Optical Properties:** the optical properties are part of the electromagnetic, and one can also say aesthetic, properties of the materials. They describe the behavior of a packaging material towards the light, i.e., in the UV-visible range (380 nm - 780 nm). In fact, light and UV rays can adversely affect food, even if for longer times than the action of oxygen or humidity, causing aesthetic and organoleptic changes and establishing degradation processes in food. To evaluate these properties, standardized measurements of sharpness are usually carried out, with the measurement of the refractive index, of transparency which is influenced by the homogeneity of the material, the degree of crystallization, and the presence or absence of particles, and depends on the thickness at which it is inversely proportional [220,221]. The optical properties can be modified, for example, by using multilayer films or additives and dyes to increase the protection of light-sensitive foods. On the other hand, protection implies fewer visual checks on the packaged food product, which is important for the consumer. Fossil polymers have high characteristics of sharpness and transparency unlike biopolymers which are opaquer due to the presence of impurities. However, these considerations obviously depend on the manufacturing technique, as techniques such as casting allow to obtain transparent materials, while techniques such as spinning inevitably lead to opaque materials. Furthermore, as already pointed out for the other properties, the use of nanofillers or multilayers to improve the chemical and physical performance of the biopolymers will affect the transparency properties [222].
- **Biodegradability and Degradability:** the importance of these chemical properties of packaging materials has assumed considerable importance only in recent times and for environmental reasons. As reported in paragraph 1.3.5, the term biodegradability means the complete degradation of material up to mineralization, i.e., only water, carbon dioxide and mineral salts by biological action, usually by microorganisms. Degradability, on the other hand, means the structural alteration of the material due to environmental factors with consequent loss of

functionality and use, or by biological agents, namely biodeterioration. Usually, chemical additives are used in plastics to avoid degradation or biodeterioration phenomena [223]. Polymers of fossil origin are particularly recalcitrant to the attack of microorganisms due to their high molecular weight, the ramifications of the polymer chain that constitute a steric hindrance, and their hydrophobicity, without forgetting the presence of additives, resulting in their almost zero biodegradability and low degradability. In the case of biopolymers, on the other hand, the problem does not arise, except that, for the production of active packaging with antimicrobial action, if on the one hand, these serve to reduce the possible contamination of food, once their use is finished it remains to be evaluated the action of these antimicrobial compounds on biodegradability. In general, if an antimicrobial active material is well designed, it should not have the presence of amounts of end-of-life antimicrobial compounds such as to inhibit biodegradability, because these compounds will have to be migrated in a controlled and authorized manner onto the food during its life shelf-life [224]. To evaluate these properties, reference is made to generally standardized procedures, such as weighting methods for determining the percentage reduction in the weight of the material subjected to the action of bacteria or by burying with the so-called Soil Burial Test; methods of microbial growth, for which the tested material represents the only source of carbon for the growth of microorganisms; respirometric methods, through the analysis of biological oxygen demand (BOD) and the measurement of the oxygen consumed or the carbon dioxide developed in the system [225,226].

2 DEVELOPMENT OF ZEIN-BASED POLYMERIC FILMS FOR ACTIVE FOOD PACKAGING

PURPOSE

The purpose of this section was to produce biodegradable antimicrobial and antioxidant polymeric films to study their potential application as active food packaging components. The main challenge of this section was posed by the choice of materials, all derived from natural sources, and by the process techniques. Indeed, the solvent casting technique was selected among the conventional ones and the electrospinning technique, considered the most innovative in the world of packaging, for the production of films with different structures and characteristics was adopted. Since both techniques require the use of solvents, also in this case green solvents recognized as GRAS (Generally Recognized as Safe) by the FDA were chosen. In particular, the polymer to which this section is dedicated is zein, a prolamin extracted from corn which, as reported in paragraph 1.3.5.1, seems to have the potential to be used as a polymer for the production of food packaging, even edible. Initially, the best process conditions were studied and identified for obtaining structures with a satisfactory morphology for both techniques. The behavior of the zein supports produced was then studied, firstly, with the addition of vanillin, chosen as the target molecule for the known antimicrobial action, which was loaded through the use of different techniques, electrospinning, solvent casting and supercritical fluids impregnation. Subsequently, the loading of a natural extract obtained with non-conventional extraction techniques from spent coffee grounds, a set of molecules more complex than vanillin, was carried out. The goal was to compare the morphology of the films obtained and the release kinetics of the active agents from the different zein films through migration tests conducted in food simulants to identify which types of food and for which modalities of conservation the films produced may be suitable. Finally, other properties such as oxygen barrier and mechanical properties were investigated comparing the sample production techniques.

2.1. Production and morphological optimization of zein films by electrospinning and solvent casting techniques

Part of the data that will be discussed in this section has been already published:

- R. Campardelli, M. Pettinato, E. Drago, P. Perego. Production of Vanillin-Loaded Zein Sub-micron Electrospun Fibers for Food Packaging Applications. *Chemical Engineering & Technology,* 2021, 44, No. 00, 1-8. Doi: 10.1002/ceat.202100044.

- R. Campardelli, E. Drago, P. Perego. Biomaterials for Food Packaging: Innovations from Natural Sources. *Chemical Engineering Transaction,* 2021, 87, 571-576. Doi: 10.3303/CET2187096.

In this section, zein films were produced using two different techniques, electrospinning and solvent casting, with the aim of producing two different polymeric structures. In fact, with the electrospinning technique, it is possible to obtain nanostructured films composed of fibers and, as such, opaque and with a large ratio between surface and volume. Whereas, through solvent casting it is possible to obtain continuous isotropic and transparent films. In particular, after identifying the polymer concentrations and the most suitable solvent, the operating conditions of the two processes were investigated to obtain morphologically satisfactory structures, verified by means of scanning electron microscope. The best set of parameters was found to be, in the case of electrospinning, a solution with a zein concentration between 20% and 35% w/w in ethanol 80% v/v, processing 10 mL of solution by applying a voltage of 17 kV, a flow rate of 1.2 mL/h, using a 21-gauge needle, a plate collector and a needle-collector distance of 16 cm. Under these conditions it was possible to obtain sub-micrometric and micrometric fibers with a mean diameter from 0.70 μm to 1.62 μm. While, through solvent casting, transparent and continuous films were obtained starting from a 20% w/w solution of zein with the addition of glycerol as plasticizer, spreading 2.5 mL of solution on a 9 cm of diameter plate and with a drying time of 2 h at 60 °C.

2.1.1 Materials and methods

2.1.1.1 Solution Preparation

The solutions to be processed by electrospinning and solvent casting were prepared starting from the literature review (paragraph 1.3.5.1). Zein is very soluble in alcoholic solutions, therefore, using the ternary phase diagram of zein, water and ethanol at room temperature of Figure 9, a hydroalcoholic solution of ethanol was chosen 80% by volume, with ethanol supplied by Carlo Erba Reagents, (Milan, Italy). The zein, supplied by Sigma-Aldrich (Milan, Italy), used without further purification, was dissolved in the solvent at three different concentrations, 20%, 25%, and 35% w/w at room temperature using a magnetic stirrer for about 1 h.

2.1.1.2 Electrospinning Process

The laboratory scale electrospinning apparatus purchased from Spinbow (Bologna, Italy), schematized in Figure 23, consists of a high voltage electrical power supplier (PCM series, Spellman, NY, USA), a syringe pump (KDS-100, KD Scientific, Holliston, MA, USA), a needle (18 and 21-gauge blunt ends were chosen, corresponding to 1.27 and 0.83 mm of outer diameter respectively)

and a metal collector (a static plate collector and a drum were chosen). The instrumentation is placed under a ventilated hood which reduces the risk of explosions due to the accumulation of flammable solvent vapors, in this case 80% v/v ethanol. In fact, the electric field could constitute a source of ignition for pure ethanol which has a flash point of 13 °C and about 25 °C for the 80% hydroalcoholic solution used.

The zein solutions were electrospun using 10 ml of polymer solution, using as starting conditions those suggested by Ferrari et al., 2017 [227]. In particular, among the parameters that govern the electrospinning process, it was decided to investigate the influence of the polymer concentration on the process, the influence of the size of the injection needle and the type of collector. In the case of the plate collector, a flow rate of 1.20 ml/h, a voltage of 17.0 kV, and a distance between the collector needle of 16.0 cm were used. When the drum collector was used, a translation speed of 600 mm/min and a rotation speed of 500 rpm was adopted.

At the end of the electrospinning process, the samples were placed in a desiccator before being analyzed. All experiments were repeated at least in duplicate.

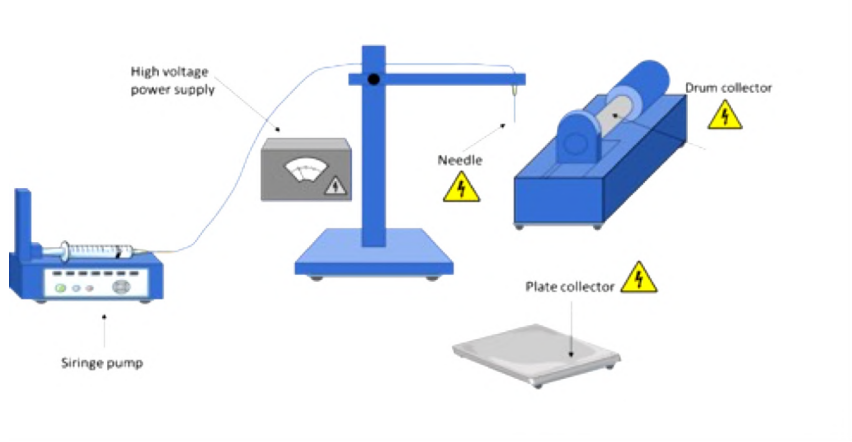


Figure 23: Schematic representation of the electrospinning apparatus. Adapted from Medical Servier Art (https://smart.servier.com/, accessed 19/02/2020).

2.1.1.3 Solvent Casting Process

As regards the solvent casting process conducted in laboratory scale as shown in Figure 24, the solutions were prepared as reported in paragraph 2.1.1.1. In addition, solutions prepared with a slight modification were also tested: a plasticizer was added, glycerol (> 99.5%, liquid) supplied by Sigma-Aldrich (Milan, Italy) equal to 30% by weight with respect to the polymer content in solution. The solutions, with a concentration of zein varying between 20 and 35% w/w, were processed by pouring 2.5 mL of each solution on glass Petri dishes with an internal diameter of 9 cm. Different ways of evaporating the solvent were also used including evaporation at room temperature with ventilation,

evaporation at room temperature without ventilation, and in the oven at 60 °C until a dry film was obtained. The dry samples were removed from the Petri dishes and stored in a desiccator before being analyzed. All experiments were repeated at least in triplicates.

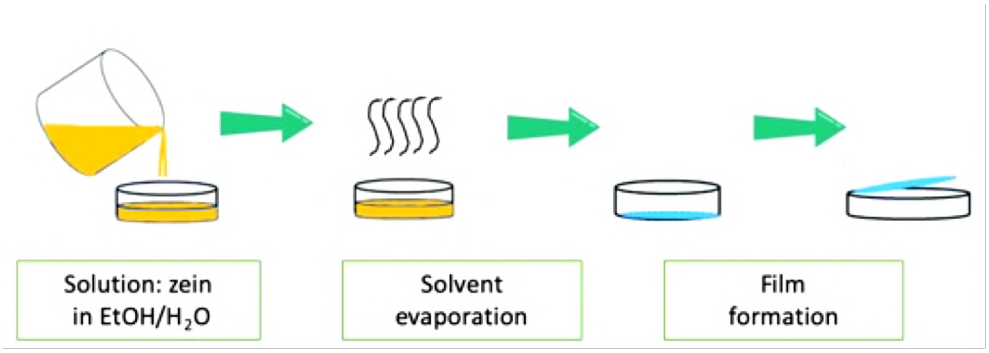


Figure 24: Schematic representation of the solvent casting process.

2.1.1.4 Morphological characterization

The samples obtained by the two techniques were morphologically characterized by Field Emission-Scanning Electron Microscope (FE-SEM, LEO 1525, Carl Zeiss SMT AG, Germany), for which samples were sputter-coated with gold thin layer before being analyzed. Furthermore, in the case of the elctrospun samples, Sigma Scan Pro 5.0 (Jandel scientific, San Rafael, QC, Canada) and Origin 9.1 (Microcal, Northampton, MA, US) were used to determine the average diameter of the fibers and to calculate the size distributions. Approximately 300 fiber diameters were measured for each calculation. Finally, the thicknesses of the best films produced with the two techniques were measured using an optical microscope (Olympus BX51) with at least five measurements for each sample.

2.1.2 Results and discussion

2.1.2.1 Zein films produced by electrospinning

A first feasibility study was conducted to evaluate the processability of the zein solution by electrospinning evaluating the influence of the collector type (fixed plate and drum) on the morphology of the samples produced. To do this, the concentration of zein in the hydroalcoholic solution of ethanol at 80% v/v was set at 25% w/w, the intermediate value between the three concentrations chosen. The electrospinning process was conducted using a voltage of 17 kV, a flow rate of 1.2 mL/h, a distance between the needle and the collectors of 16 cm, and a 21-gauge needle.

The electrospinning process was successful and the fibers homogeneously covered the entire surface of the collectors. FE-SEM images of the produced zein fibers are shown in Figure 25.

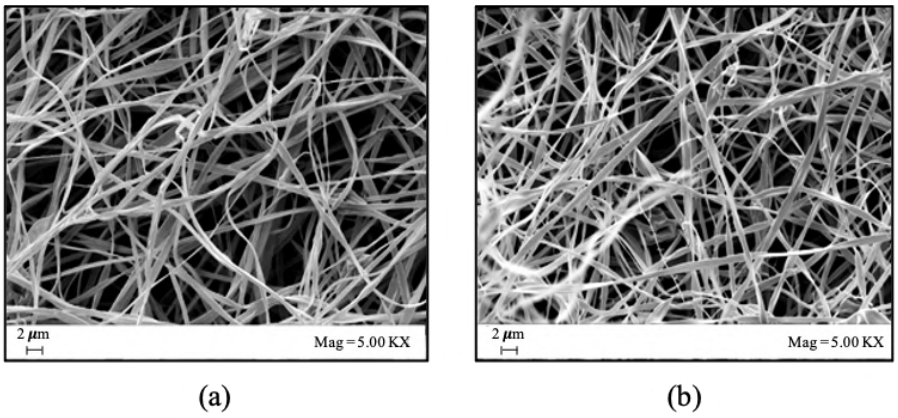


Figure 25: FE-SEM images of zein 25% w/w fibers collected using: (a) drum collector, and (b) plate collector.

The fibers appeared well formed, without defects, with the typical random orientation that gives the general structure of the non-woven mats. The mean fiber diameter, determined as described in the "Materials and Methods" paragraph of this section, was 0.83 ± 0.25 µm using the drum collector and 0.70 ± 0.22 µm using the plate collector. A comparison of the distribution curves of the size of the fibers as the collector varies is shown in Figure 26.

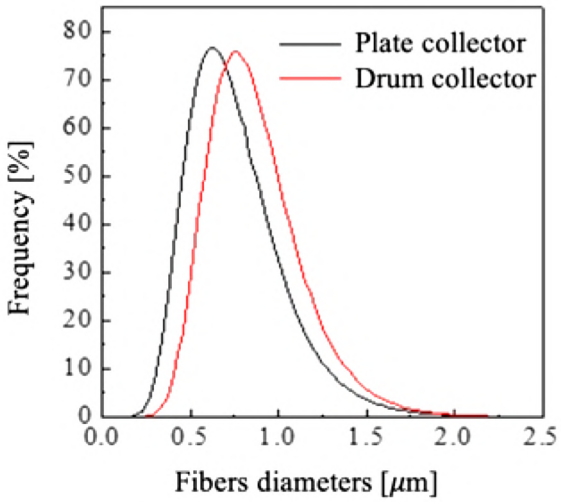


Figure 26: Comparison of the zein fibers size distribution curves as the collector varies.

From Figure 26, it can be seen that the dimensions of the fibers do not vary significantly with the type of collector. Considering the final application for packaging materials, it was decided to continue the experiments using the plate collector as a planar geometry is preferable for film making, such as the one shows in Figure 27.

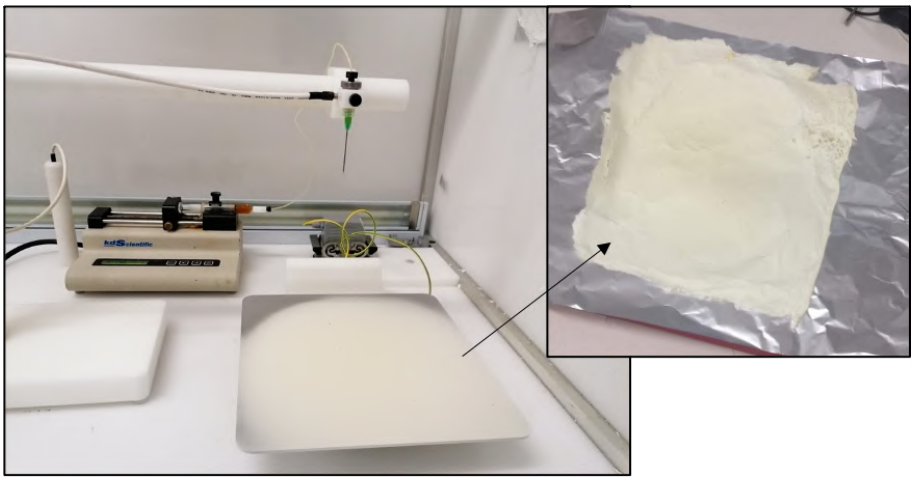


Figure 27: Formation process of the electrospun zein film on the plate collector.

Once the processability of zein was verified by electrospinning, it was decided to investigate the effect of zein concentration on the diameter of the fibers. For this reason, zein solutions at the concentration of 20 and 35% w/w were also processed.

Zein fibers were collected successfully also for the 20% and 35% w/w solution concentration. FE-SEM images of the obtained fibers are reported in Figure 28. It can be seen that finer, more filiform fibers but with some superficial irregularity, were obtained for the lowest concentration, 20% w/w (Figure 28a), with a mean fiber diameter of approximately 0.71 ± 0.24 μm with no particular difference with respect to the 25% w/w concentration of zein. Only by increasing the concentration of zein to 35% w/w, a substantial increase in the mean diameter of the fibers is obtained, with a more ribbon-like shape, already evident from the FE-SEM image of Figure 28b, with a mean fiber diameter equal to 1.62 ± 0.45 μm.

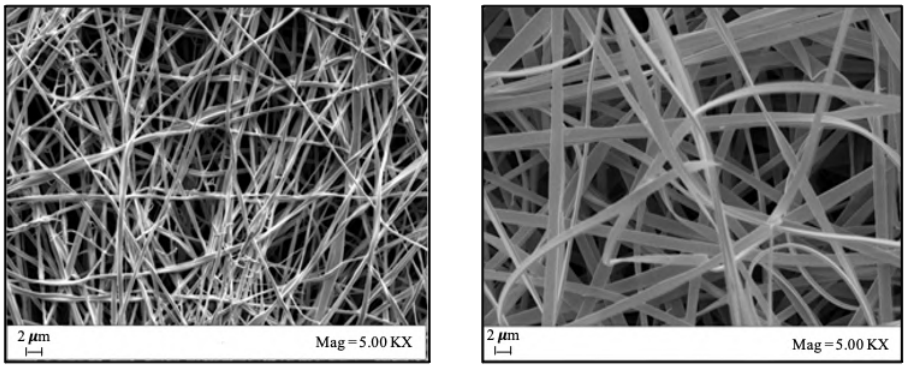


Figure 28: FE-SEM images of fibers produced from a 20 % w/w (a) and 35 % w/w zein concentration in the ethanol 80% v/v.

In order to better highlight the effect of zein concentration on fibers diameter, a comparison of the fiber distribution curves is reported in Figure 29.

The effect of concentration onto fibers size is attributable to the increase in the viscosity of the polymeric solution which has an impact on the electrodynamic process as also confirmed by other authors [228].

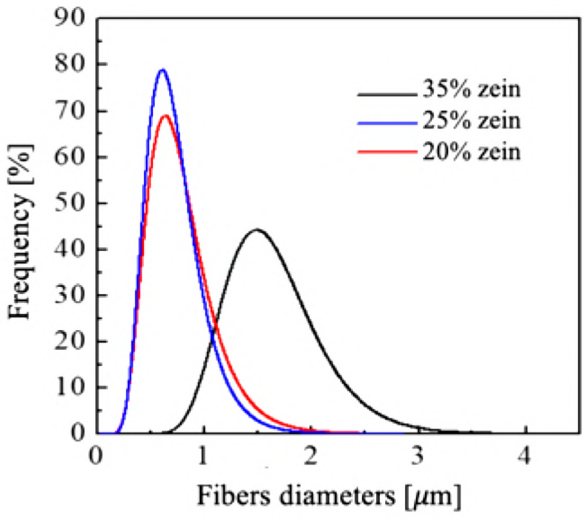


Figure 29: Comparison of the zein fibers size distribution curves as the polymer concentration (%, w/w) varies.

Finally, the influence of needle size on fiber morphology was investigated. Therefore, taking the case at 25% w/w as reference zein concentration, the electrospinning tests were repeated using a larger 18-gauge needle (1.27 mm). As reported in Figure 30, there was no variation in the mean diameter of the fibers, which was found to be equal to 0.72 ± 0.23 μm, with a distribution that was perfectly superimposable to the tests conducted with the 21-gauge needle.

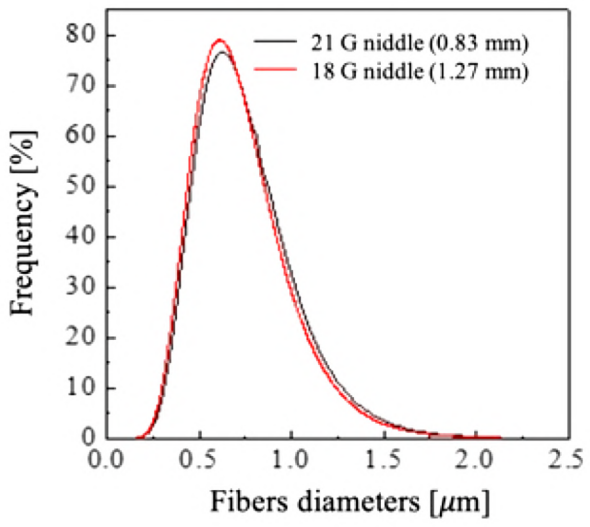


Figure 30: Comparison of the zein fibers size distribution curves as the needle gauge varies.

The conduct of these latter experiments may seem trivial, but, as reported by Correira et al., 2014 [229], the scientific literature has reported contradictory results on the effect of needle size on fibers dimension. The results discussed in this section were in agreement with the investigation conducted by Macossay et al. [230], who concluded that the mean fiber diameter does not depend on this parameter. However, according to the authors cited above, the size of the needle could affect the polydispersion of the curve, but the results of the present study showed no effect on the fiber size distribution curve, using the selected needle size. Other authors have provided opposite conclusions [231]. Furthermore, the thickness of the films obtained did not depend on any parameter but rather on the process time. Therefore, for all concentrations of zein, for an average electrospinning time of 6 hours, films with a mean thickness of 54 ± 7.51 μm were obtained, however very variable, for all tests, depending on the measurement point on the surface of the films. In fact, on the edges the films were thinner, up to 30% less than in the central area. Therefore, the diameter of the fibers is a better reproducibility parameter than the thickness. To summarize the results of this part, Table 7 shows

the tests carried out by electrospinning and the relative mean diameters expressed as mean value ± standard deviation.

Table 7: Summary of the electrospinning tests carried out by varying zein concentration, type of collector and needle size, with relative mean diameters (MD) and standard deviations (SD) of the obtained fibers.

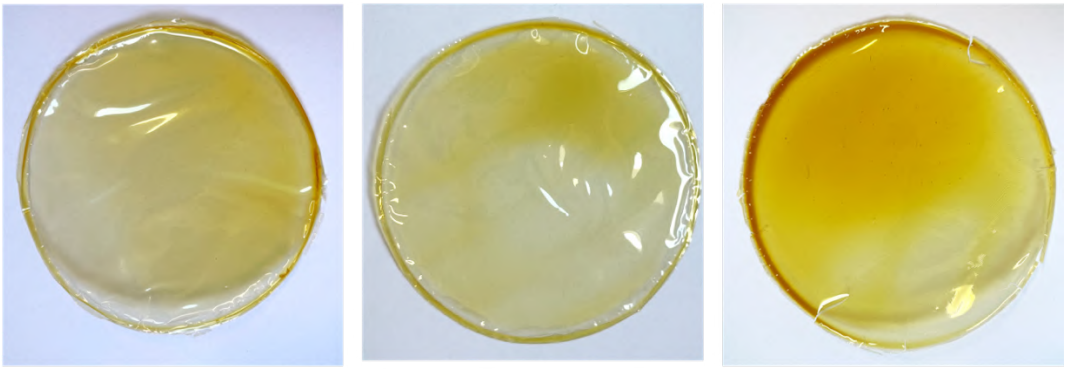
Zein concentration [%, w/w]	Collector Type	Needle [gauge]	MD ± SD [µm]
25	Drum	21	0.83 ± 0.25
25	Plate	21	0.70 ± 0.22
25	Plate	18	0.72 ± 0.23
20	Plate	21	0.71 ± 0.24
35	Plate	21	1.62 ± 0.45

Considering the good results obtained from these tests, it was not considered necessary to carry out other tests with other polymer concentrations or other operational process parameters.

2.1.2.2 Zein films produced by solvent casting

For the zein samples produced by solvent casting, the need to use a plasticizer was immediately evident given the impossibility of removing the films from the Petri dishes once the solvent evaporated. The polymer solutions were then modified by adding glycerol, chosen among the various plasticizing agents to keep the formulation of the constructs as green as possible and food compatible, at 30% w/w with respect to the zein content, dissolved in the solution. Different concentrations of zein were tested, as in the case of electrospinning, this time starting from concentrations below 20% w/w, but it was not possible to obtain any type of film due to the scarcity of polymer present in the solution. The tests were then carried out at 20%, 25%, and 35% w/w of zein, up to concentrations higher than 35% w/w, but above this limit, the solution becomes too viscous to be spread on the Petri dishes uniformly. The range of polymer concentration identified for electrospinning was therefore confirmed again for this technique, the best one. Once the possible concentrations of zein were identified, the ways of solvent evaporation were investigated. In particular, for a casting volume of 2.5 mL, the samples were placed at room temperature with ventilation, at room temperature without ventilation, and in the oven at 60 °C until a dry film was obtained. The best tests were found to be those carried out in an oven at 60 °C for which about 2 hours were required to obtain dry zein films which are easily removable from the plates. The tests carried out at room temperature, on the other hand, did not give good results, in the sense that it was not possible to remove the films from the

supports completely, despite the presence of glycerol, because they showed a high fragility as well as, in the case of the presence of ventilation, to surface defects such as waves. Therefore, the evaporation of the solvent in an oven at 60 °C was selected as the most suitable condition. Higher temperatures have not been tested to avoid denaturing the protein and also with a view to subsequently loading of compounds sensitive to high temperatures. Furthermore, evaporation times that are too fast would lead to the formation of defects and air bubbles and therefore holes in the films. Some images of the films obtained by varying the concentration of zein, with the addition of glycerol at 30% with respect to the weight of zein, pouring 2.5 mL onto 9 cm Petri dishes, and leaving them in the oven at 60 °C for 2 h are reported in Figure 31.



(a) (b) (c)

Figure 31: Zein film produced by solvent casting pouring 2.5 mL: (a) zein 20% w/w, (b) zein 25% w/w and (c) zein 35% w/w.

As can be seen, through solvent casting it was possible to obtain transparent films, yellow in color due to the color of the commercial zein used, which increases in intensity as the concentration of zein increases.

However, the samples produced at the higher concentrations, i.e., 25% and 35% w/w, showed greater stiffness and brittleness to handling than the case of zein at 20% w/w, making their analysis difficult. Therefore, the concentration equal to 20% by weight was chosen as the best to produce manageable films, with a thickness comparable to that of the electrospun samples. In fact, the average thickness of the films produced at 20% w/w of zein was found to be about 51 ± 9.46 μm, up to a double thickness for the concentrations of zein equal to 35% w/w. The thicknesses of the films produced were not uniform, as evidenced by the irregularity of the color of the samples shown in Figure 31; this is due to the manual spreading of the solutions on the supports which did not allow a homogeneous control of the thickness as instead is ensured by the process on an industrial scale.

The morphology of the 20% zein samples analyzed by FE-SEM is shown in Figure 32 from which it is possible to observe the presence of some superficial small irregularities probably due to the solvent evaporation phase, but the structural difference with respect to electrospun supports is very clear.

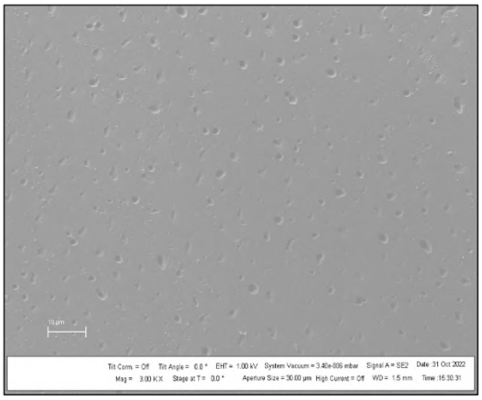


Figure 32: FE-SEM images of film produced by solvent casting from a 20 % w/w zein concentration in the ethanol 80% v/v.

The polymeric structures obtained through the two techniques were therefore found to be satisfactory and suitable to be replicated for the subsequent step of loading active agents with antimicrobial and/or antioxidant action as discussed in the next paragraphs.

2.2 Vanillin loading as antimicrobial agent: techniques comparison

The data that will be discussed in this section has been already published:

- R. Campardelli, M. Pettinato, E. Drago, P. Perego. Production of Vanillin-Loaded Zein Sub-micron Electrospun Fibers for Food Packaging Applications. *Chemical Engineering & Technology,* 2021, 44, No. 00, 1-8. Doi: 10.1002/ceat.202100044.
- R. Campardelli, E. Drago, P. Perego. Biomaterials for Food Packaging: Innovations from Natural Sources. *Chemical Engineering Transaction,* 2021, 87, 571-576. Doi: 10.3303/CET2187096.
- E. Drago, P. Franco, R. Campardelli, I. De Marco. Zein electrospun fibers purification and vanillin impregnation in a one-step supercritical process to produce safe active packaging. *Food Hydrocolloids,* 2022, 122. Doi: 10.1016/j.foodhyd.2021.107082.

This section was dedicated to the study of a phenolic aldehyde loading, vanillin, the main component of vanilla beans, known as a flavoring compound but which also has high antimicrobial action, for example against *Escherichia Coli* and *Saccharomyces cerevisiae*. The vanillin was loaded directly, through its encapsulation in the polymer during the film production phase by electrospinning and solvent casting, and indirectly, after the production of the electrospun films by supercritical fluids impregnation (SFI), for which the tests were conducted at the Department of Industrial Engineering of the University of Salerno, Italy. The impregnation of active compounds by SFI seems to be an interesting green alternative to producing active packaging without requiring solvents or high temperature. The samples obtained were again characterized in terms of morphology to investigate the influence of the vanillin presence. To verify the actual vanillin loading in the polymer, the encapsulation efficiency was evaluated, which was found to be approximately 74-76% in the electrospun and impregnated fibers for any vanillin load-tested independently on the vanillin load. In the case of solvent casting, the encapsulation efficiencies were between 85% for the lower load and equal to 65% in the case of the upper vanillin load. In order to verify the active packaging migratory character, migration tests were conducted in different food simulants by comparing the migration kinetics obtained with the three techniques. The results showed how it is possible to modulate the migration kinetics as needed, by varying the production technique and the concentration of the loaded agent. Considering, the migratory behavior and the antimicrobial activity successfully tested on *Saccharomyces cerevisiae*, the samples produced showed the possibility of being used as an active component in packaging, for example on dried fruit and baked goods for the aromatic imprint of vanillin, as film in the case of solvent casting and as patches to be inserted in the primary packaging in the case of electrospun films.

2.2.1 Materials and Methods

2.2.1.1 Direct loading of vanillin by electrospinning

Zein fibers loaded with vanillin (purity 99%) purchased by Sigma Aldrich, Milan, Italy, were produced by dissolving a fixed amount of vanillin in the zein solution at 25% and 35% w/w concentration to obtain different theoretical loading of 5, 10 and 15% w/w with respect to zein content. The electrospinning process was carried out using the operating parameters identified in the previous section, i.e., a voltage of 17 kV, a flow rate of 1.2 mL/h, and a distance between the needle and the plate collector of 16 cm. Each experiment was conducted at least in triplicate.

2.2.1.2 Direct loading of vanillin by solvent casting

The solvent casting technique was also used to produce the antimicrobial film. The solution was prepared by dissolving zein with 20% w/w concentration in ethanol 80% v/v, stirred at room temperature until a complete dissolution was achieved. At this point, vanillin was added, from 5 to 15% w/w with respect to the zein, and 0.24 mL of glycerol was also added as plasticizer. Finally, the prepared solutions were poured onto Petri dishes (2.5 mL for each plate) which were then placed in an oven at 60 °C for 2 h before being peeled and analyzed. Each experiment was conducted at least in triplicate.

2.2.1.3 Indirect loading of vanillin by supercritical fluids impregnation

The impregnation of active agents using the supercritical carbon dioxide (scCO2) is a solvent-free process conducted under mild conditions of $scCO_2$ (T_c= 31.1 °C, P_c= 7.38 MPa). The process is governed by the diffusive capacity of $scCO_2$ in polymeric supports which allows compounds soluble in it to penetrate thanks also to its ability to swell the polymeric supports, improving diffusion. Thanks to the high diffusivity of $scCO_2$, efficient penetrations can be obtained. The porous polymeric supports seem to be particularly suitable for being impregnated with $scCO_2$, i.e., electrospun sample, although, to the best of our knowledge, it was attempted in very few papers, none of these for food packaging applications [232,233].

The supercritical impregnation of vanillin into the fibrous support were performed in a homemade laboratory plant of the Department of Industrial Engineering of the University of Salerno, Italy, sketched in Figure 33.

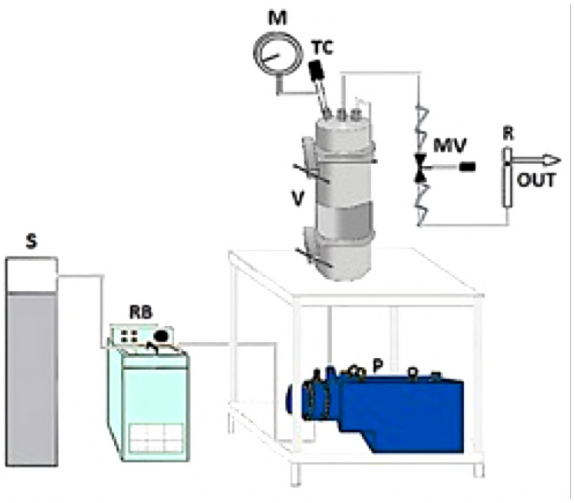


Figure 33: Laboratory plant for fibers' purification and vanillin adsorption by $scCO_2$. S: CO_2 supply; RB: refrigerating bath; P: pump; V: autoclave; MV: micrometric valve; TC: thermocouple; M: manometer; R: rotameter [234].

It consists in an autoclave (NWA GmbH, Germany) with a 100 mL internal volume, closed with two finger tight clamps. The CO_2 (99% purity), provided by Morlando Group s.r.l., Sant'Antimo, Naples, Italy, is cooled before the compression through a refrigerating bath; therefore, it is powered by a diaphragm piston pump (Milton Roy, mod. Milroyal B, France). Homogeneous mixing in the autoclave is ensured by an impeller, mounted on the top cap. A digital pressure gauge (Parker, Minneapolis, MN) measures the operating pressure inside the vessel, while a PID controller (Watlow, mod. 93, USA) ensures thermal control. Finally, a micrometric valve (Hoke, mod. 1315G4Y, Spartanburg, SC) allows the system to be depressurized. The CO_2 flow is measured by a rotameter placed at the exit of the autoclave. The amount of CO_2 supplied to the autoclave is determined by the density value, which is calculated at the operating temperature and pressure using the Bender equation of state [235]. This process was first used by placing the electrospun samples of zein alone in contact with the supercritical fluid to investigate its effect. After identifying the optimal process conditions, the vanillin impregnation tests were performed using a static method. In short, a known amount of vanillin (20 mg) was loaded into a small container mounted axially on the impeller open on the top to allow contact between the compound and the supercritical fluid. A known amount of electrospun support (2.5cm x 2.5cm) was placed on the bottom of the autoclave. The CO_2 was then slowly pumped into the heated vessel up to 40 °C and 50 °C. Once the pressure of 17 MPa is reached, the CO_2 supply is interrupted and the contact of the support with the $scCO_2$ is maintained for a determined time. At the end of the test, the $scCO_2$ is slowly brought back to atmospheric conditions, with a constant flow rate of about 0.1 MPa/min. The weight and area of the fibrous supports were measured before and after the process to determine any changes due to contact with $scCO_2$. Each test was repeated at least in triplicate.

2.2.1.4 Morphological characterization

Fiber morphology was analyzed using a Field Emission-Scanning Electron Microscope (FE-SEM, LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany), coating the samples with a gold-palladium layer by a sputtering coater (thickness 250 Å, model 108 A, Agar Scientific, Stansted, UK). Sigma Scan Pro 5.0 (Jandel scientific, San Rafael, QC, Canada) and Origin 9.1 (Microcal, Northampton, MA, US) were used to determine the mean fibers diameter and to evaluate the size distributions. Approximately 300 fibers were analyzed for each calculation.

2.2.1.5 Vanillin encapsulation efficiency

Vanillin present into zein structure after the processing was determined by dissolving a fixed amount of zein film (approximatively 5 mg) into 5 mL of ethanol 80% v/v. Vanillin concentration was analyzed via UV-vis spectrophotometer (model Lambda 25, Perkin Elmer, Wellesley, MA) at 280 nm. After the construction of the calibration curve, vanillin concentration (C) was obtained from the measurement of absorbances using the Lambert-Beer law reported in equation (1).

$$ABS = \varepsilon \times b \times C \quad (1)$$

Where: ABS = absorbance read by the instrument; ε = molar absorption coefficient [$L*cm^{-1}*mol^{-1}$]; b = optical path of the solution [cm]; C = concentration of the solution [$mol*L^{-1}$]; ε*b = angular coefficient of the calibration line.

In this way the amount of vanillin really present into zein films can be calculated. The real loading is therefore the ratio between the mass of vanillin measured and the weight of the zein samples used for the analysis. The encapsulation efficiency (EE %) was determined according to the equation (2):

$$EE\ (\%) = {}^{Real\ loading}/_{Theoretical\ loading} \quad (2)$$

Vanillin encapsulation efficiency tests were performed in triplicate.

2.2.1.6 Vanillin migration tests

The migration of vanillin from the zein films was conducted by total immersion migration method in food simulants. For this purpose, zein film specimens (2.5cm × 2.5cm) were weighted and totally submerged in the respective simulant medium (15 mL) in glass bottles, sealed tightly, in order to have a surface to volume ratio between 0.5 and 2 cm^2/cm^3 as recommended by Ministerial Decree 21 March 1973, Annex IV [236]. Migration tests were carried out, depending on the production technique, both on distilled water and two food simulants: 10% v/v ethanol, for food products with

hydrophilic features, and 3% w/v acetic acid solution for food products with hydrophilic features and pH < 4.5, as recommended by the European Regulation n 10/2011 on plastic materials and articles intended to come into contact with food (European Commission, 2011). The test conditions adopted were those provided for by the Regulation for products with a short shelf-life to be stored at room temperatures (such as fresh cut fruit), for which tests are expected to be carried out in the worst conditions of mass transfer, that is, in in this case, 37 °C. After 24 hours of static contact, vanillin in the food simulant was analyzed using the UV-vis spectrophotometer at 280 nm. Vanillin migration tests were conducted in triplicate.

2.2.1.7 Thermal analysis

Thermal analysis was conducted using a Differential Scanning Calorimeter (DSC, mod. TC11, Mettler-Toledo, Inc., Columbus, USA) on the electrospun and impregnated samples to investigate the thermal behaviour after the supercritical process. The samples were weighted (approximately 5 mg) and heated from 25 to 200 °C at 10 °C/min in nitrogen atmosphere (50 mL/min).

2.2.1.8 Antimicrobial activity of zein films loaded with vanillin

The antimicrobial activity of vanillin was tested against *Saccharomyces cerevisiae* by measuring its growth over time in the presence of vanillin contained in the zein films.

This yeast was chosen as the exponent of the class of eukaryotic microorganisms. It is a non-pathogenic yeast whose uncontrolled and unwanted growth can induce food spoilage. The growth was determined in liquid by optical density (OD) measurements at 650 nm [237]. *Saccharomyces cerevisiae* was firstly placed in the YPD broth (LLG Labware, Meckenheim, Germany) and incubated overnight at 37 °C under shaking (250 rpm) in aerobic conditions before its use. A known quantity of each sample, both zein films produced by casting and by electrospinning unloaded and loaded with the three percentages of vanillin, 5%, 10% and 15% w/w, was placed in contact with a known volume of medium containing the yeast to maintain constant the ratio between the sample weight and the culture volume. The samples were incubated under shaking at 37 °C and the *S. cerevisiae* growth was observed by spectrophotometric analysis at 650 nm at regular intervals from 30 minutes until the yeast death phase was reached. A culture medium containing the yeast was used as a control sample. The tests were repeated in duplicate.

2.2.1.9 Statistical Analysis

Statistica v8.0 software (StatSoft, Tulsa, OK, USA) was used to perform a statistical evaluation of experimental data. Analysis of variance (ANOVA) and Tukey's post-hoc test was carried out to

assess the significance of differences among groups with statistical significance considered at a probability value (p) < 0.05.

2.2.2 Results and discussion

2.2.2.1 Vanillin loading by electrospinning

The operating parameters identified in chapter 2.1 were left unchanged, i.e., a voltage of 17 KV, a flow rate of 1.2 mL/h, a needle-collector distance of 16 cm and a 21-gauge needle, while the concentration of zein and the vanillin theoretical load were systematically varied. The vanillin loading effect was evaluated by producing samples using both the 25% w/w and 35% w/w zein hydroalcoholic solution. The vanillin was dissolved in different quantities in order to obtain the 5, 10, and 15% w/w theoretical load with respect to the zein content. The operating conditions and the main results in terms of mean fiber diameter (MD), real loading, and encapsulation efficiency (EE) are shown in Table 8. The results are expressed as mean value ± standard deviation (SD).

Table 8: Operative parameters of electrospinning of zein loaded with vanillin by varying zein and vanillin concentration. Fibers mean diameter (MD), vanillin theoretical and real loading and vanillin encapsulation efficiency (EE) are also reported. Different symbols indicate statistically significant differences among different theoretical loading ($p<0.05$) of dataset with the same zein concentration.

Zein concentration [% w/w]	**Theoretical loading [% w/w]**	**Needle [gauge]**	**MD ± SD [µm]**	**Real loading [% w/w]**	**EE [%]**
25	5	21	0.71 ± 0.24	3.65 ± 0.26	72.9 ± 6.0
25	10	21	0.72 ± 0.23	7.13 ± 0.07	71.3 ± 0.70
25	15	21	0.67 ± 0.24	11.3 ± 0.64	75.0 ± 4.2
35	5	21	1.73 ± 0.47	3.78 ± 0.17	75.6 ± 3.5
35	10	21	1.81 ± 0.53	7.41 ± 0.56	74.1 ± 5.6
35	15	21	1.82 ± 0.52	11.3 ± 0.99	75.5 ± 6.6

The fibers were successfully collected for each set of experiments reported in Table 8, from which it is possible to observe that as the theoretical vanillin load increased, the mean diameters remained practically constant with a negligible variation. Starting with the case of 25% zein, it is possible to note that produced fibers with zein 25% w/w are sub-micrometric, with random orientation and no significant variation between the loaded and the un-loaded fibers. The mean diameter of the zein alone was found to be equal to 0.70 ± 0.22 µm; with the addition of vanillin, the mean diameter was

between 0.71 ± 0. 24 μm to 0.67 ± 0.24 μm. The maintenance of the morphological structure of the fibers was confirmed in FE-SEM images shown in Figure 34 (a-c) for the sample produced at 5, 10, and 15% of the vanillin load with 25% zein concentration and in Figure 34 (d-f) for the sample produced at 5, 10, and 15% vanillin load with 35% zein concentration.

Instead, the set of experiments performed at higher zein concentration, 35% w/w, was characterized by micrometric fibers with mean diameter in the range between 1.73 ± 0.47 μm and 1.82 ± 0.52 μm, with mean fiber diameter not significantly affected by vanillin loading variation. For both zein concentrations, no statistically significant difference was found in terms of mean diameter as the vanillin load varied. As also observed for the experiment for production of empty zein fibers, the 35% w/w of zein concentration in polymer solution resulted in an increase in fibers dimension from sub-micrometric to micrometric size. Instead, the thickness of the samples remained around a constant value of 60 ± 6.4 μm. Looking at Figure 34 (d-f), it can be observed that a more pronounced ribbon-like structure is evident in the samples produced at higher zein concentration. This effect was also observed for fibers produced with only zein.

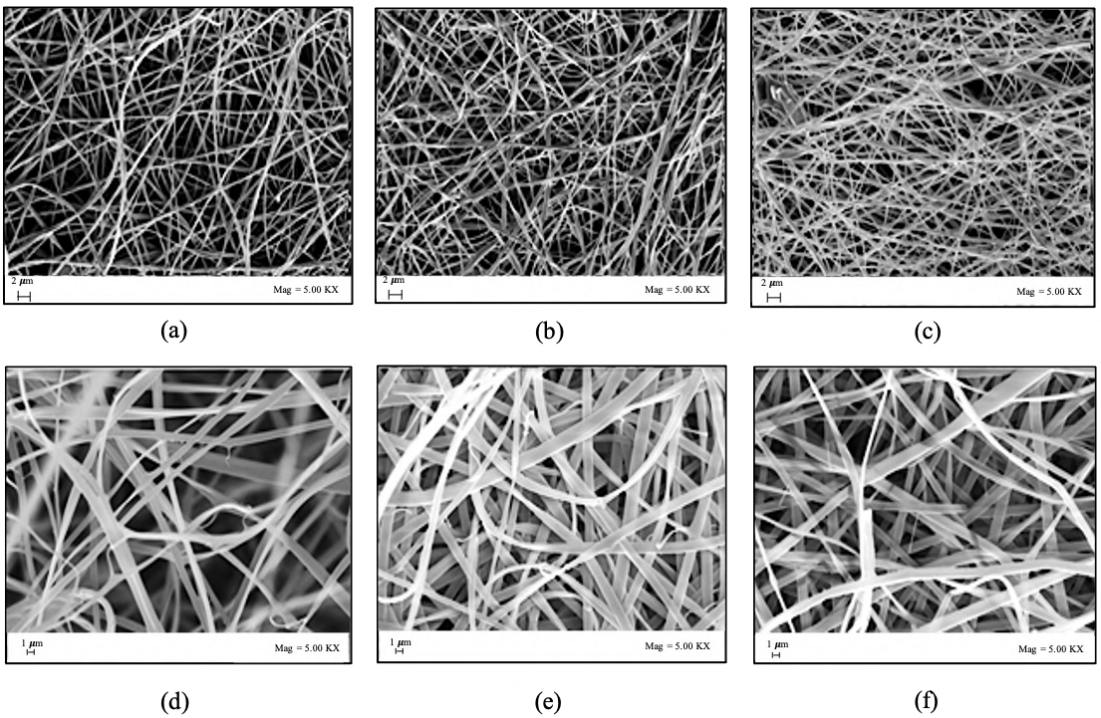


Figure 34: FE-SEM images of zein-vanillin fibers produced at different vanillin loadings: (a) 5 % w/w, (b) 10 % w/w, (c) 15 % w/w with zein 25% w/w concentration; (d) 5 % w/w, (e) 10 % w/w, (f) 15 % w/w with zein 35% concentration.

So, it can be concluded that the presence of vanillin did not affect fibers mean diameter and morphology. Furthermore, from the morphological analysis it is possible to make another consideration: in all the samples no vanillin crystals precipitated outside the fibers were detected, indicating that, most likely, the vanillin was co-precipitated with the zein during the process of fiber formation. The confirmation of the incorporation of vanillin in the zein fibers was obtained from the UV-vis analyses for the determinations of the encapsulation efficiency, carried out as described in the "Materials and Methods" paragraph of this section. The efficiency of vanillin encapsulation in zein fibers was between 71% and 75% for both concentrations of zein and for all theoretical loadings of vanillin with no statistically significant difference. This value could be the effect of a systematic loss of vanillin during the process steps such as solution preparation, amount of solution remaining into the tubing of the plant and a volatilization of vanillin with ethanol evaporation during fibers formation. Another possible explanation of vanillin loss can be the oxidation of vanillin induced by the solvent and electrospinning process conditions. Indeed, it is reported that vanillin can degrade in conditions of temperature, light and presence of reactive species [238,239]. This contribute seems to be constant and responsible to about 26% loss of active compound. When a polymer and a compound are processed together, basically two different scenarios can be considered. In the first situation, the polymer and the compound precipitate separately due to the different crystallization kinetics. In this condition the presence of crystals of the compound could be noticed on the outside of the polymeric fibers. In the second case, however, the precipitation rates are comparable and the compound is captured by the polymer molecules, which are generally also the most abundant. If this condition occurs, almost all of the compound loaded in the initial solution is actually present in the final structure. Therefore, since no vanillin crystals are visible outside the fibrous structure in the fiber mats produced, the amount of vanillin loss during the process may only be due to the volatilization or degradation of the vanillin during the process.

Finally, the vanillin migration tests from the zein films were investigated by the total immersion migration method. The food simulants selected were distilled water, 10% v/v ethanol in water and 3% w/v acetic acid in water from those requested by the European Regulation n 10/2011 [181]. The tests were conducted by immerging specimens of about 12.5 cm^2, considering the double face exposed to the simulant, into 15 mL of food simulant for 24 hours after which the release liquids were analyzed by UV-vis spectrophotometry at 280 nm to quantify migrated vanillin. The samples investigated in this paragraph were those produced exclusively by electrospinning of zein at 25% w/w with vanillin loaded at the three target concentrations of 5, 10 and 15% w/w, with the aim of study the effect of the different simulants. The results obtained are summarized in Table 9 and expressed in terms of percentage of vanillin released and in terms of specific migration, i.e., mg of vanillin per cm^2 of sample.

Table 9: Migration of vanillin from zein 25% w/w films into distilled water, acetic acid 3% w/v (A.A.3%) and ethanol 10 % v/v as food simulants at 37 °C after 24 h. Different symbols indicate statistically significant differences among different theoretical loading ($p<0.05$) of dataset with the same simulant.

Theoretical vanillin loading [%, w/w]	**Real vanillin loading [%, w/w]**	**Simulant**	**Specific migration [$mg_{vanillin}/cm^2$]**	**Vanillin release [%]**
5	3.65 ± 0.26	water	0.25 ± 0.02	81.1 ± 9.1
10	7.13 ± 0.07	water	0.19 ± 0.02	69.7 ± 9.7
15	11.26 ± 0.64	water	0.36 ± 0.05	79.5 ± 11.9
5	3.65 ± 0.26	A.A. 3%	0.17 ± 0.01	63.2 ± 4.8
10	7.13 ± 0.07	A.A. 3%	0.33 ± 0.06	69.0 ± 11.6
15	11.26 ± 0.64	A.A. 3%	0.50 ± 0.12	73.0 ± 18.9
5	3.65 ± 0.26	EtOH 10%	0.16 ± 0.01	89.6 ± 5.6
10	7.13 ± 0.07	EtOH 10%	0.46 ± 0.12	72.7 ± 19.3
15	11.26 ± 0.64	EtOH 10%	0.39 ± 0.08	63.8 ± 14.7

From Table 9, it is possible to observe that as the vanillin load increases, its migration in distilled water has not a specific trend, but the average value of migration is almost the same for all the loading, passing from 81.1 ± 9.1 % to 79.5 ± 11.9 % as demonstrated by statistical analysis carried out that evidenced no statistically difference among the values. As regards the tests conducted in 3% w/v acetic acid, considering the mean values, it is possible to observe a trend, that is, as the vanillin load increases, the percentage migration increases, passing from a value of 63.16 ± 4.8 % to 73.07 ± 18.9 %, but no statistically significant difference was highlighted by ANOVA analysis due to the high standard deviation obtained. Instead, a different behavior was observed for ethanol 10% v/v for which the percentage release of vanillin was found to be maximum in correspondence of the lower vanillin load and then decreased by increasing the vanillin load in the zein fibers, passing from 89.6 ± 5.6 % to 63.8 ± 14.7 % considering the mean values, but again, no statistically significant difference was highlighted by ANOVA analysis due to the high standard deviation. The EE% mean values obtained with ethanol 10% v/v may probably be due to the affinity of ethanol with zein. In fact, zein is soluble in hydroalcoholic solutions. In fact, a visible swelling of the polymeric structure was observed after 24 h of contact with the simulant. The plasticizing effect of the simulant on the film

surface probably creates a barrier to the diffusion of vanillin. However, the migrations are all quite high considering only the 24 hours of testing. Furthermore, it can be deduced that for food products with a hydrophilic character and lipid content, it might be more convenient to use films produced with the maximum load of vanillin in order to have a release over time as prolonged as possible. Instead, for hydrophilic and acidic food products, it may be better to use films with a lower vanillin load for the same reason. In any case, all films produced by electrospinning may have the potential to be applied as antimicrobial patches to be inserted inside a primary packaging given their opacity and can only be used for foods with a short shelf-life.

At the end of this paragraph, the results of the migration tests conducted on zein at 25% w/w in the three different simulants are summarized in graphic form in Figure 35.

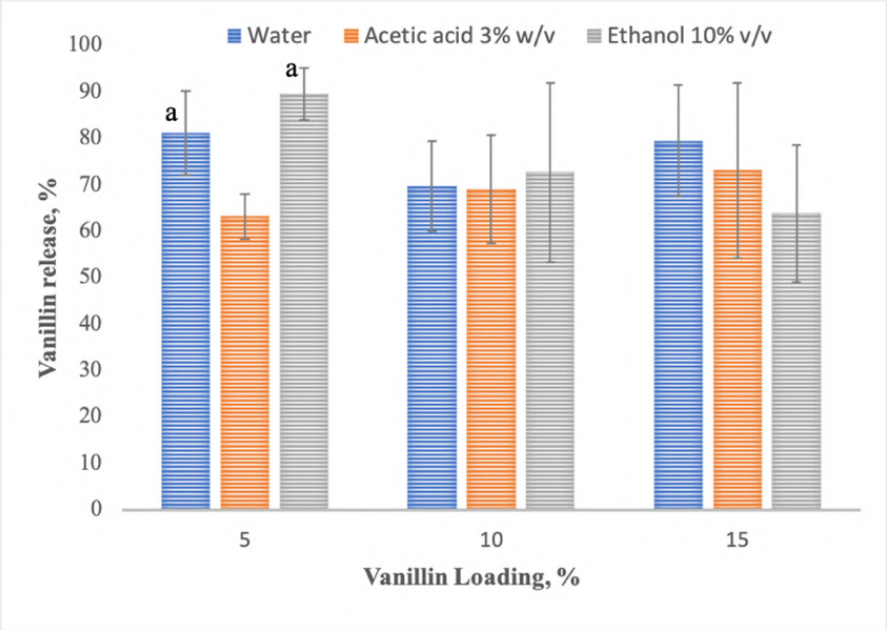


Figure 35: Migration of vanillin from zein films produced at 25% w/w zein into different food simulants at 37 °C after 24 h of contact. Different symbols indicate statistically significant differences among values as simulant variation ($p<0.05$) of dataset with the same vanillin loading.

2.2.2.2 Vanillin loading by solvent casting

The loading of vanillin into zein was also carried out using the solvent casting technique, using the operating parameters identified as the best in chapter 2.1. Therefore, vanillin, as in the case of electrospinning, was loaded with a theoretical load equal to 5%, 10%, and 15% w/w with respect to the zein content, in order to be able to conduct a comparison between these two techniques. For all the loads, the films were uniform without defects and therefore ready to be characterized. The film

thickness was found to be quite variable on single samples of the same type, but the average thickness when the vanillin concentration changed, expressed as mean value ± standard deviation, was found to be quite constant as shown in Table 10.

The migration tests were carried out in ethanol 10 % v/v as food simulant, at 37 °C for 24 h of contact. The release liquids were then analyzed, as described in the previous paragraph, by UV-vis spectrophotometry at 280 nm. The results showed that the percentage migration of vanillin was minimal in the samples with the lower vanillin load and then showed a constant but significantly higher trend with increasing loading. These results were obtained assuming that the encapsulation efficiency of vanillin in films produced by solvent casting was 100%, being a process with little possibility of material loss. It is interesting to compare the results obtained with those already analyzed in the same simulant in the case of the electrospinning technique. From the graph shown in the Figure 36, it can be seen that the two different polymeric structures have a completely opposite behavior in terms of migration of vanillin into 10% v/v ethanol.

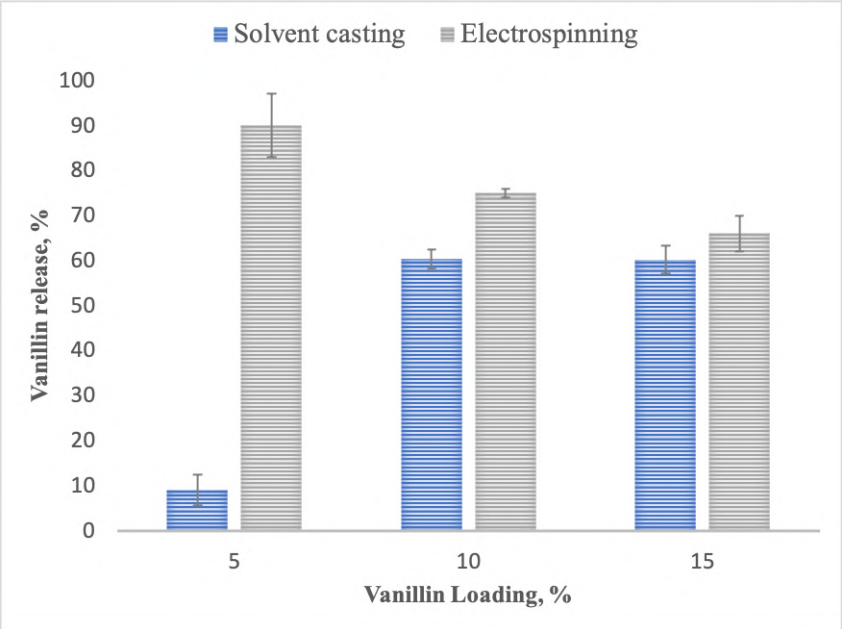


Figure 36: Vanillin migration (%) from zein film produced by solvent casting and electrospinning in ethanol 10% v/v as food simulant conducted at 37 °C for 24 h.

In fact, with the minimum vanillin load equal to 5% w/w, the samples produced by electrospinning with zein 25% w/w, showed a practically total migration of vanillin in 24 h, while the samples produced by solvent casting showed a very little migration and an increase of the vanillin load allowed an increase in the migration reaching a value of about 60%. This clear difference between

the samples produced by solvent casting, and, above all, the lower migration compared to the electrospun films, is to be attributed to the surface exposed by the samples to the simulant. In fact, in the case of solvent casting, the exposed surface is much lower than that of the fibers, therefore the swelling action of the simulant is reduced and the kinetics of migration is therefore also reduced, which becomes proportional to the quantity of active compound loaded. Whereas, in the case of electrospinning, the high surface exposed by the fibers, and the porous nature, make the structure highly attackable by the simulant with consequent greater migration, especially if the active compound is present in small quantities.

However, from the evaluation of the encapsulation efficiency, it emerged that this was found to decrease as the vanillin load increased as reported in Table 10. In fact, encapsulation efficiencies were obtained with an average of 85% in the case of the vanillin load of 5% w/w, and then passing to a 76% in the case of vanillin at 10% w/w up to reach a 64% in the case of vanillin loaded at 15% w/w.

Table10: Results of zein loaded with vanillin by solvent casting in terms of film thickness, real loading and vanillin encapsulation efficiency (EE%) expressed as mean value ± standard deviation.

Zein concentration [%, w/w]	**Theoretical loading [%, w/w]**	**Thickness [µm]**	**Real loading [% w/w]**	**EE [%]**
20	5	52 ± 12.3	4.24 ± 0.36	85.0 ± 7.2
20	10	46 ± 4.3	7.60 ± 0.56	76.1 ± 5.6
20	15	40 ± 9.6	9.57 ± 0.10	63.9 ± 0.6

This behavior confirms what was hypothesized in the previous paragraph for electrospinning, namely that vanillin can degrade in unfavorable temperature and light conditions. In this case, probably, the exposure of the samples to 60 °C led to a degradation of the active compound which was greater in the case of the higher load since, the quantity of vanillin dissolved in zein, tends to distribute itself superficially in the volume exposed to evaporation with consequent loss both by evaporation and by degradation. Therefore, the migration test results were re-analyzed based on these encapsulation efficiencies. The graph with the percentage release of real vanillin as the load varies is shown in the Figure 37.

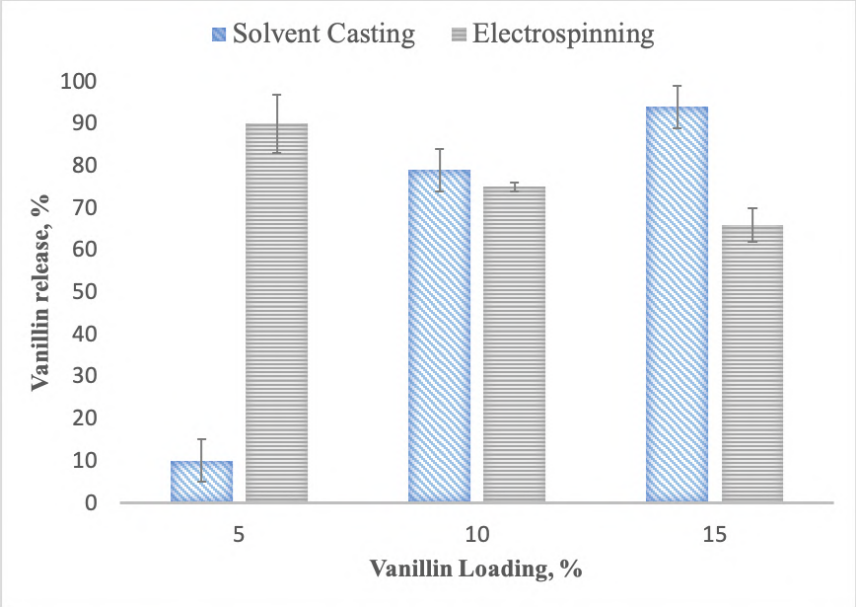


Figure 37: Vanillin release (%) comparison between solvent casting and electrospinning considering the real encapsulation efficiency.

As can be seen, compared to an ideal encapsulation efficiency equal to 100%, also in this case the migration trend of vanillin is the same but with a higher percentage increase in migration as the vanillin load increases. This result allows concluding that in order to have high releases in a few hours it is possible to use electrospun films with low concentrations of active compound, or transparent films produced by casting with high concentrations of the active compound. Or again, to have prolonged releases over time, the best solution is offered by castings loaded with low concentrations of the active compound.

2.2.2.3 Vanillin loading by supercritical fluids impregnation

To carry out a feasibility study of vanillin loading on electrospun zein support by supercritical fluids impregnation (SFI), and also to study the effect of the contact of electrospun films with supercritical carbon dioxide, the samples were again produced by electrospinning for all three concentrations of zein identified in chapter 2.1, i.e., 20%, 25% and 35% w/w. The feasibility tests of the vanillin impregnation into zein fibers were conducted on samples with reduced size (2.5 cm x 2.5 cm) for a contact time fixed to 24 h, a temperature of 40 °C and a pressure of 17 MPa. For each electrospun support, the vanillin uptake was expressed as mg of impregnated vanillin per mg of zein fibers. The experimental data obtained are reported in Table 11.

Table 11: Feasibility tests of vanillin impregnation into different fibrous support at 40 °C, 17 MPa and an impregnation time of 24 h.

Zein Concentration [% w/w]	**$mg_{vanillin}/mg_{zein}$ [% w/w]**
20	0.39
25	0.97
35	1.97

From the data, it is possible to observe an increase in the impregnated vanillin load as the concentration of zein increases, and therefore the mean diameter of the fibers. This result is probably due to the better formation of zein fibers obtained at the higher concentration, as observable from the FESEM images shown in Figure 38. The lower coalescence of the support certainly favors the supercritical impregnation of vanillin.

FESEM analyzes were performed on all samples both before and after supercritical impregnation. Observing FESEM images of the sample at 20% w/w concentration of zein (Figure 38a), a partial deterioration of the structure can be observed after the contact with the $scCO_2$ (Figure 38d). For the 25% and 35% w/w zein supports, the structure was preserved after the supercritical process and vanillin crystals trapped in the fibrous structure are visible (Figure 38b, 38e and Figure 38c, 38f).

Therefore, considering that the greatest amount of vanillin was impregnated in the support produced at the highest concentration of zein and that this also maintained the structure of the fibers unlike in the case of 20% w/w zein, the support at 35% w/w was selected as the best for the supercritical impregnation of vanillin and used for the next steps of the work.

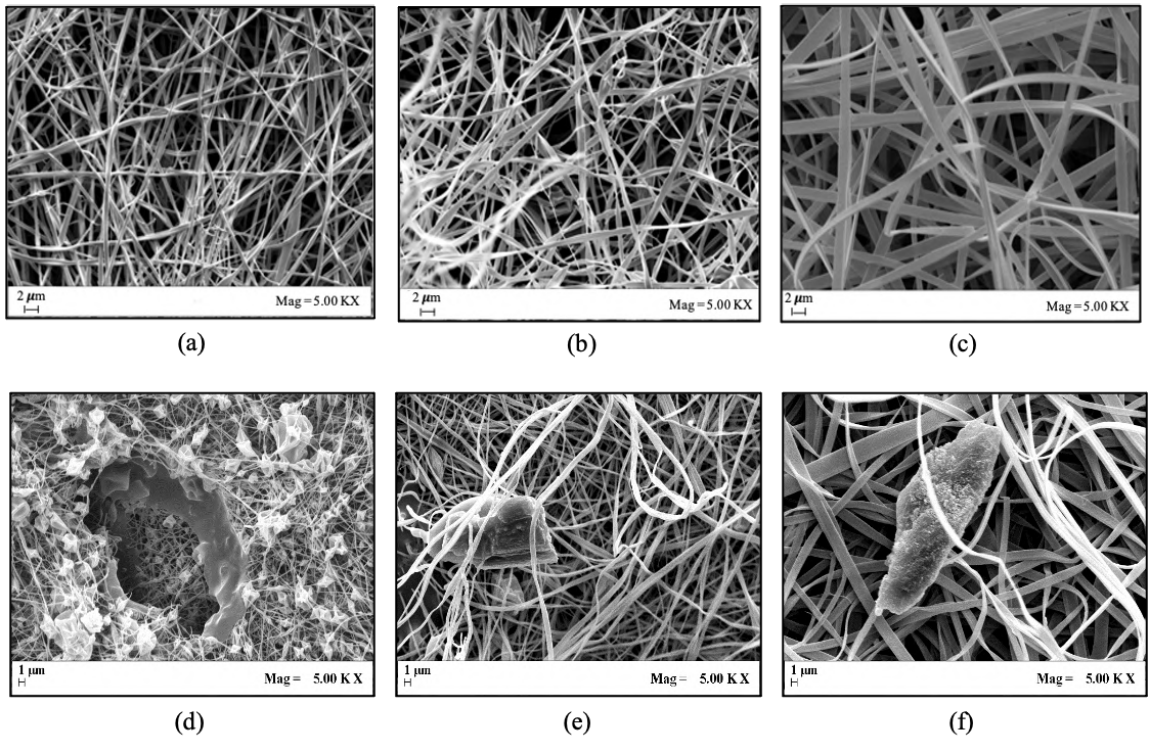


Figure 38: FESEM images of zein electrospun supports before $scCO_2$ process: (a) 20% w/w; (b) 25% w/w; (c) 35% w/w; and after vanillin impregnation with $scCO_2$ process conducted for 24 h, 40 °C and 17 MPa: (d) 20% w/w; (e) 25% w/w; (f) 35% w/w.

Furthermore, it was observed that all samples subjected to contact with $scCO_2$ showed a significant reduction in surface and weight and a loss of the yellow color intensity of the zein films after contact with $scCO_2$. This effect could be due to the extraction of the residual solvent and the lipophilic compounds from the zein samples. To confirm this hypothesis, a study was conducted on vanillin-free zein films only. The zein samples were placed in contact with the supercritical fluid at the previous conditions of temperature equal to 40 °C and pressure equal to 17 MPa, this time varying the contact time between 6 and 24 h. The samples, with initial dimensions equal to 2.5 x 2.5 cm, were weighed before and after the process to quantify the weight loss and the areas of the film before and after contact with $scCO_2$ were measured to evaluate the shrinkage effect.

The experimental data relating to the percentage weight loss of the zein fibers in contact with $scCO_2$ during time are reported in Table 12. The percentage of mass variation of the sample calculated before and after the process is also reported. It can be observed that, regardless of the process time, an average reduction of the mass of the support after supercritical process of about 24% was obtained.

Table 12: Study of zein contact with $scCO_2$ at 40 °C, 17 MPa. Determination of the weight loss (%) and sample shrinkage at different contact times. W_i is the weight of electrospun zein fibers before the supercritical processes; W_f is the fibers' weight after the contact; W_d is the weight difference; A_i is the area of the support before the process; A_f the is the area after the process and Δarea is the variation of the area of the support after supercritical process.

Time [h]	**W_i** [mg]	**W_f** [mg]	**W_d** [mg]	**Weight loss** [%]	**A_i** [cm^2]	**A_f** [cm^2]	**Δ_{area}** [%]
6	22.4	16.4	6.0	26.8	6.25	5.04	19.4
15	43.7	36.0	7.7	17.6	6.25	5.28	15.5
24	22.7	16.5	6.2	27.3	6.25	4.62	26.1

The area reduction (%) reported in Table 12, was calculated as follows in equation (3):

$$\Delta area\ (\%) = {}^{(Ai - Af)}/_{Ai} \times 100 \quad (3)$$

The percentage reduction in the area increased from 19.4% to 26.1% as the hours of contact with the supercritical fluid increased. Considering also the relative weight loss and surface reduction, it can be stated that during contact with $scCO_2$ an extraction of residual solvents and also of non-polar compounds present in the zein matrix with good solubility in $scCO_2$ is obtained, such as example waxes and lipids present in the zein as impurities in the processing. Therefore, in the face of the tests conducted so far, it was possible to conclude that the supercritical impregnation on the zein fibers has a double effect: the impregnation of the active compound and the purification of the fibers from residual solvents and lipophilic compounds.

Considering these results satisfactory, the work was continued with a systematic study for the optimization of the impregnation process. As a first step, the impregnation kinetics was determined, in order to know the time required to reach equilibrium, that is the maximum quantity of impregnated vanillin. To do this, vanillin was impregnated for contact times between 2 and 48 h, at a pressure of 17 MPa and evaluating two different temperatures, 40 and 50 °C, to also study the effect of temperature on the kinetics. The experimental data obtained are reported in Table 13, while the resulting impregnation kinetics obtained at the two temperatures are reported in Figure 39.

Table 13 . Experimental kinetics data at 40 °C and 50 °C at various contact time.

T [°C]	time [h]	$mg_{vanillin}/mg_{zein}$ [%, w/w]	T [°C]	time [h]	$mg_{vanillin}/mg_{zein}$ [%, w/w]
40	2	0.61	50	2	4.30
40	4	1.04	50	4	3.57
40	8	1.19	50	8	10.62
40	15	1.96	50	24	11.18
40	24	1.97	50	48	11.20

The percentage of vanillin loaded was expressed as mg of vanillin per mg of zein support (%) and was evaluated by UV-vis analysis at 280 nm. It can be seen that the amount of loaded vanillin increases significantly with the process temperature. This result can be attributed to the increase in the solubility of vanillin in $scCO_2$ with temperature. In particular, the maximum quantity of vanillin impregnated in the support was reached between 24 h and 48 h at 50 °C.

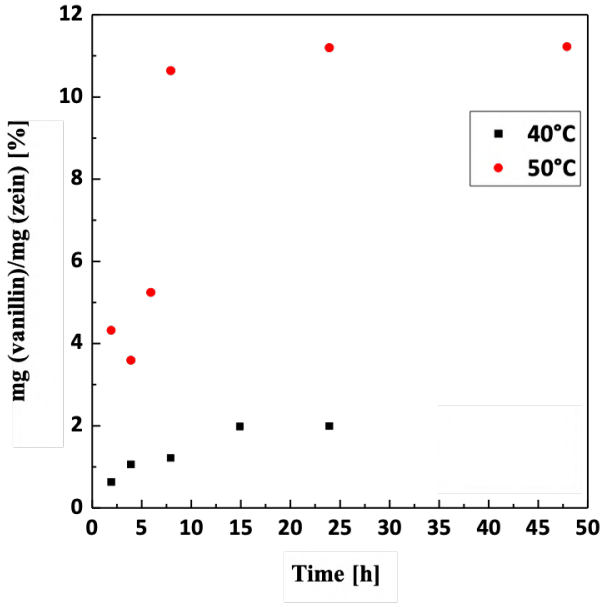


Figure 39: Kinetics curves at 40 °C and 50 °C, and 17 MPa, for the supercritical impregnation of vanillin into zein fibers.

In order to verify if the experimental and theoretical loadings are in good agreement and to study the specific rate constants, the kinetic data were fitted with pseudo-first-order and pseudo-second-order equations, which were obtained by integrating the following equation (4) [240]:

$$d\left(1-\frac{q_t}{q_e}\right)/_{dt} = -k\left(1-{}^{q_t}/_{q_e}\right)^n \quad (4)$$

Where q_t (mmol/g) is the amount of vanillin loaded into the zein support after the contact time, q_e (mmol/g) is the adsorption capacity at the equilibrium condition.

A comparison among the impregnation rate constants obtained from the pseudo-first-order and pseudo-second-order models is reported in Table 14.

Table 14: Impregnation rate constants obtained from the pseudo-first-order and pseudo-second-order kinetics at 40 °C and 50 °C.

T (°C)	**Pseudo-first-order kinetics**			**Pseudo-second-order kinetics**		
	q_e, mmol/g	**k_1, 1/h**	**R^2**	**q_e, mmol/g**	**k_2, g/mmol h**	**R^2**
40	0.128	0.404	0.789	0.167	0.899	0.964
50	0.736	0.261	0.931	0.846	0.198	0.965

From Table 14, it can be observed that the pseudo-second-order kinetics fitted better the experimental data compared to the pseudo-first-order kinetics, since the values of R^2 are higher. The pseudo-second-order equation (5) was expressed as:

$${}^{t}/_{q_t} = {}^{t}/_{q_e} + {}^{1}/_{k_2 {q_e}^2} \quad (5)$$

Where k_2 is the pseudo-second-order rate constant (g/mmol h).

Plotting t/q_t in function of time, q_e and k_2 were determined by the slope and the intercept of the fitting, as reported in Figure 40.

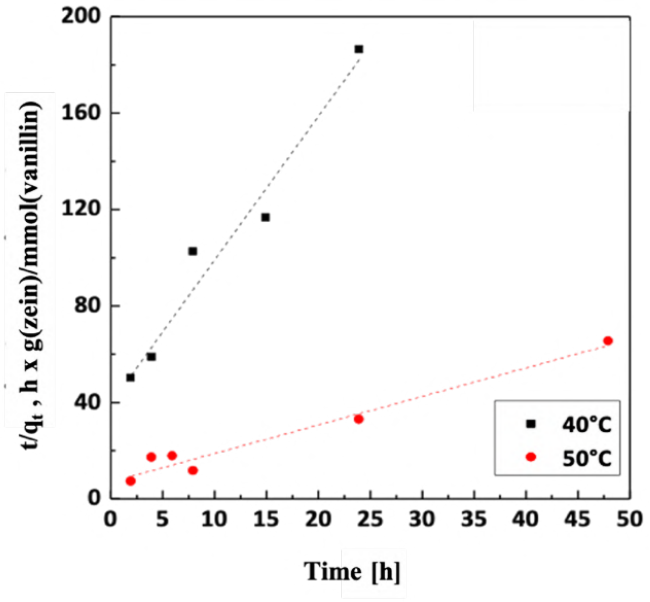


Figure 40: Pseudo-second-order kinetics for the supercritical impregnation of vanillin into electrospun zein fibers, at 17 MPa at two different temperatures.

Comparing these results with the vanillin loading obtained directly by electrospinning, the data of which have been discussed in Table 8, it is possible to note that with the SFI process the same vanillin loading can be reached for contact times equal to 2, 8 and 24 hours. The results of the vanillin loading obtained with the two techniques compared are shown in the Table15.

Table 15: Comparison between vanillin loaded into zein fibers produced directly by electrospinning considering the theoretical loading and vanillin loaded using the supercritical impregnation process considering the process time to achieve the desired loading. The zein support was always at 35% w/w concentration.

Electrospinning		***scCO₂ Impregnation***	
Theoretical vanillin load [%, w/w]	**Real vanillin load [%, w/w]**	**Process time [h]**	**Real vanillin load [%, w/w]**
5	3.78 ± 0.17	2	4.44 ± 1.2
10	7.41 ± 0.56	8	10.62 ± 0.64
15	11.33 ± 0.99	24	11.18 ± 0.89

In the case of $scCO_2$ impregnation the presence of vanillin is also detectable from FESEM images, where crystals of vanillin are visible entrapped inside the fibrous structure. This result is dependent from the different loading method adopted. Indeed, in the electrospinning process, vanillin is dissolved in the initial solution with zein and therefore, it is present in the fiber structure produced by electrospinning; whereas, in the case of $scCO_2$ impregnation vanillin is dissolved in the supercritical fluid and released into the fibrous structure. This difference can produce composite materials with different vanillin release behavior. This aspect will be analyzed in the following paragraph.

The thermal behavior of the supports obtained through these techniques was also studied by means of calorimetric analyzes conducted on pure vanillin, electrospun zein, electrospun zein processed also with $scCO_2$, vanillin impregnated by $scCO_2$ and vanillin loaded directly by electrospinning. The thermograms are shown in Figure 41. The DSC curve of vanillin showed an endothermic peak at approximately 82 °C corresponding to its melting temperature [241]. The thermograms of all fiber-based samples showed the characteristic thermal behavior of zein: the wide endothermic peak in the range of 50-150 °C attributed to dehydration and the glass transition temperature (T_g) around 170 °C [242]. Furthermore, in the thermograms of all electrospun fibers not processed by $scCO_2$, another peak was evident at about 85 °C, probably due to solvent residues or impurities as already assumed. Furthermore, as further confirmation, this peak was not detected in all samples processed in the presence of $scCO_2$, confirming the supercritical purification of the electrospun fibers. The melting peak was not observed in the DSC thermograms of all vanillin-loaded fibers by both electrospinning and supercritical impregnation, since vanillin is probably hidden in the polymer matrix.

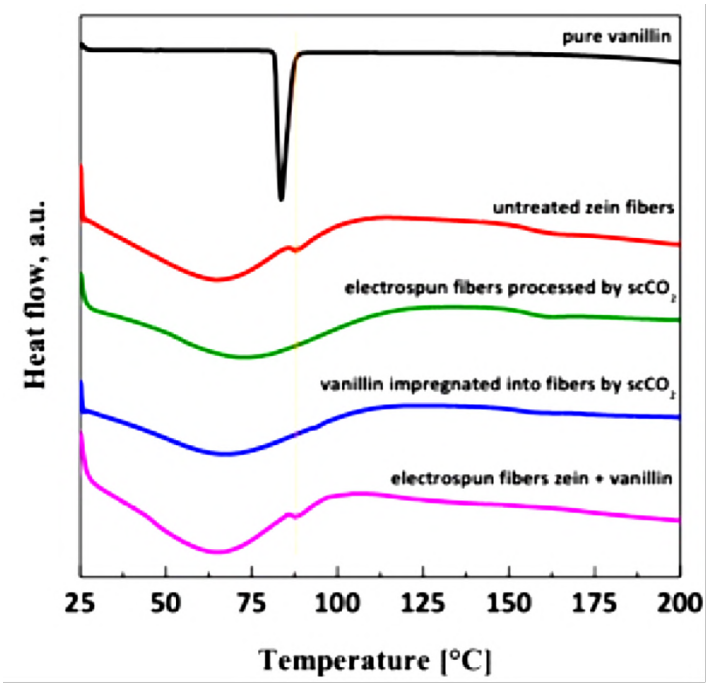


Figure 41: DSC thermograms of pure vanillin, untreated electrospun zein fibers, electrospun fibers purified by $scCO_2$ process, vanillin impregnated into zein fibers by $scCO_2$, vanillin-loaded fibers produced by electrospinning.

FT-IR spectra of pure vanillin, electrospun zein fibers, $scCO_2$-impregnated vanillin, and vanillin loaded by are shown in Figure 42. The FT-IR spectrum of pure vanillin showed many absorption bands, including those around 731, 1510 and 1590 cm^{-1} relative to the stretching vibrations of the benzene ring, and the band at about 1660 cm^{-1} relative to the C = O stretching of the aldehyde group [243,244]. The spectrum of untreated zein fibers produced by electrospinning showed the characteristic absorption bands of the polymer, such as peaks at approximately 1645 cm^{-1}, 1540 cm^{-1} and 1235 cm cm^{-1} of amide I, II and III, respectively [245]. The spectra of electrospun fibers processed in the presence of $scCO_2$, both with and without vanillin, are similar to those of untreated fibers. The spectrum of vanillin-loaded fibers prepared by electrospinning showed bands more characteristic of vanillin than supercritical impregnated samples. When the active compound was loaded by electrospinning, probably more vanillin molecules were likely to be near the surface of the single fiber, while vanillin is hidden in the fibrous polymer matrix when impregnated with $scCO_2$.

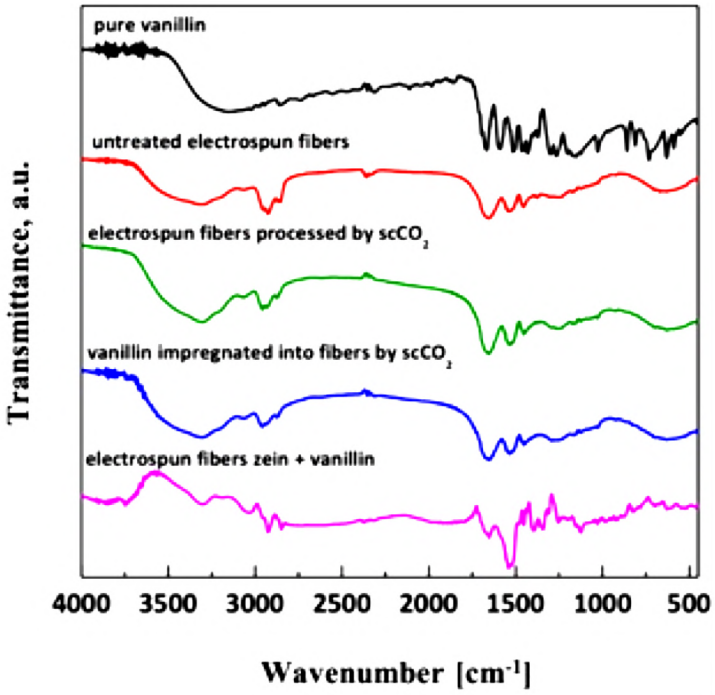


Figure 42: FT-IR spectra of pure vanillin, untreated electrospun zein fibers, electrospun fibers purified by $scCO_2$ process, vanillin impregnated by $scCO_2$, vanillin-loaded fibers produced by electrospinning.

The migration kinetics of vanillin from zein films was also investigated for $scCO_2$ impregnated samples. The tests were always conducted in ethanol 10% v/v at 37 °C for 24 h of contact on the electrospun samples of zein at 35% w/w processed by SFI for a contact time equal to at 2, 8 and 24 hours, i.e., the times that were suitable for providing a vanillin load comparable to the real load obtained by electrospinning. The results are reported in the Table 16 in which the two techniques are compared.

Table 16: Migration test of vanillin loaded zein 35% w/w fibers produced by electrospinning process with different vanillin theorical loading (sample A: 5%, sample B: 10%, sample C: 15% w/w) and $scCO_2$ impregnation at different process time (sample D: 2 h, sample E: 8 h, sample F: 24 h) carried out in EtOH 10% v/v by total immersion method at 37 °C for 24 h.

Sample	**Real vanillin load [%, w/w]**	**Specific migration [$mg_{vanillin}/cm^2$]**	**Vanillin release [%]**
		Electrospinning	
A	3.78 ± 0.17	0.11 ± 0.02	90 ± 0.4
B	7.41 ± 0.56	0.19 ± 0.01	84 ± 8.6
C	11.33 ± 0.99	0.26 ± 0.01	89 ± 7.4
		$scCO_2$ impregnation	
D	4.44 ± 1.20	0.22 ± 0.01	88 ± 7.2
E	10.62 ± 0.64	0.13 ± 0.02	71 ± 8.0
F	11.18 ± 0.89	0.12 ± 0.01	50 ± 3.3

From the data reported in Table 16 and also showed in Figure 43 for a graphic view, it is possible to note that, after 24 h of tests in contact with the food simulant, vanillin was released almost in its entirety from the electrospun samples of zein at 35% w/w concentration, as had already been discussed previously. In fact, it is possible to observe that as the vanillin load increases, the release remains at a high and rather constant value, while the amount of vanillin released per unit area increases with the increase in the vanillin load, because with the same surface area, if there is more vanillin, more can be released. Instead, the 35% w/w zein samples loaded by supercritical impregnation, showed a lower release and with a clear decreasing trend, also the specific migration per surface unit showed a decreasing trend with increasing process hours and therefore of vanillin load. This different behavior is not taken for granted considering that the polymeric supports were made using the same technique. Therefore, it is probable that it is due to the different locations of the vanillin in the zein structure as the loading technique varies. Indeed, in the case of direct loading by electrospinning, the zein is incorporated into the polymer itself and therefore is uniformly dispersed in the fibers with consequent rapid release in the simulant. While, in the case of supercritical impregnation, as was also visible from the FE-SEM images in Figure 38, vanillin is

dissolved by the $scCO_2$ and then diffused into the fibrous structure, where it is trapped in the form of crystals so that there is a slower because the simulant needs more time to dissolve.

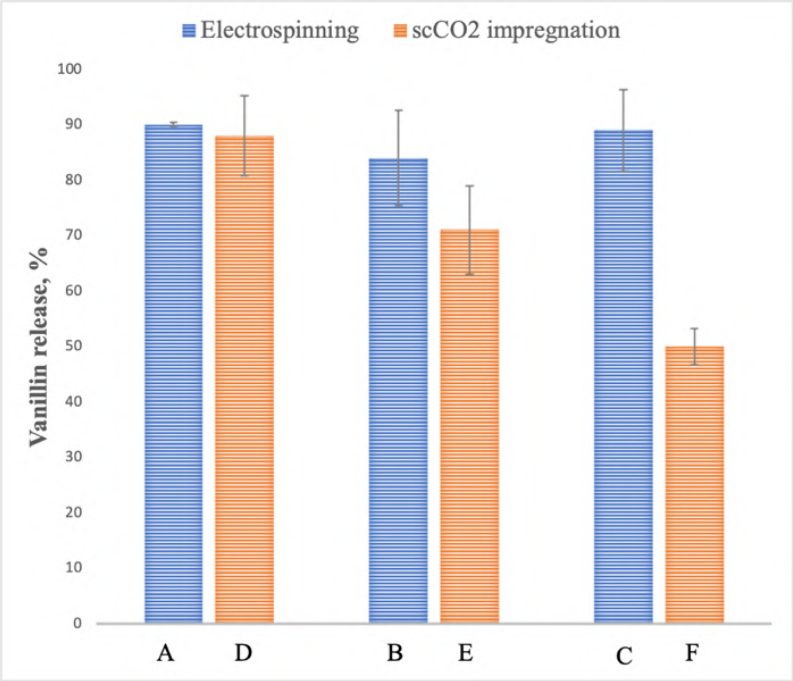


Figure 43: Vanillin % released from zein loaded fibers after 24 h of contact with EtOH 10% v/v by total immersion method at 37°C for 24 h. Electrospinning sample (A) 5%w/w, (B) 10% w/w, (C) 15% w/w and $scCO_2$ impregnated sample: (D) 2h, (E) 8h and (F) 24 h.

2.2.2.4 Antimicrobial activity of vanillin contained in zein films

The preliminary analysis of the antimicrobial activity carried out in liquid through the measurement of the optical density or the turbidity of the culture by means of spectrophotometer is based on the proportional link that exists between the measurement of optical density and the number of yeast cells present in the culture [237]. In particular, the optical density of the starting culture, measured at 650 nm, was found to be equal to 0.119 nm, corresponding to approximately 1 x 10^6 cells per mL. The analyzed samples were prepared in order to maintain the ratio between the weight of the sample, and therefore the theoretical content of vanillin, and the volume of the culture medium constant and equal to 0.010. The theoretical vanillin concentration present in the samples regardless of the technique used to produce them was, therefore: 3 mM for the samples of zein at 5% w/w of vanillin, 6 mM for the samples with 10% w/w of vanillin and 9.5 mM for the 15% w/w vanillin samples. The samples were immersed in a culture medium based on YPD broth inoculated with *Saccharomyces cerevisiae* and placed in an incubator at 37 °C under shaking to stimulate the growth of the yeast.

The culture medium containing the yeast was used as the control sample while the unloaded zein films were used as the blank sample. The culture medium of each sample was analyzed by spectrophotometer at different time intervals starting from 30 min until reaching the yeast death phase which occurred after about 26 hours. The results of the antimicrobial tests expressed in terms of optical density (OD at 650 nm) are reported in Figure 44 for the films produced by electrospinning and Figure 45 for the films produced by solvent casting.

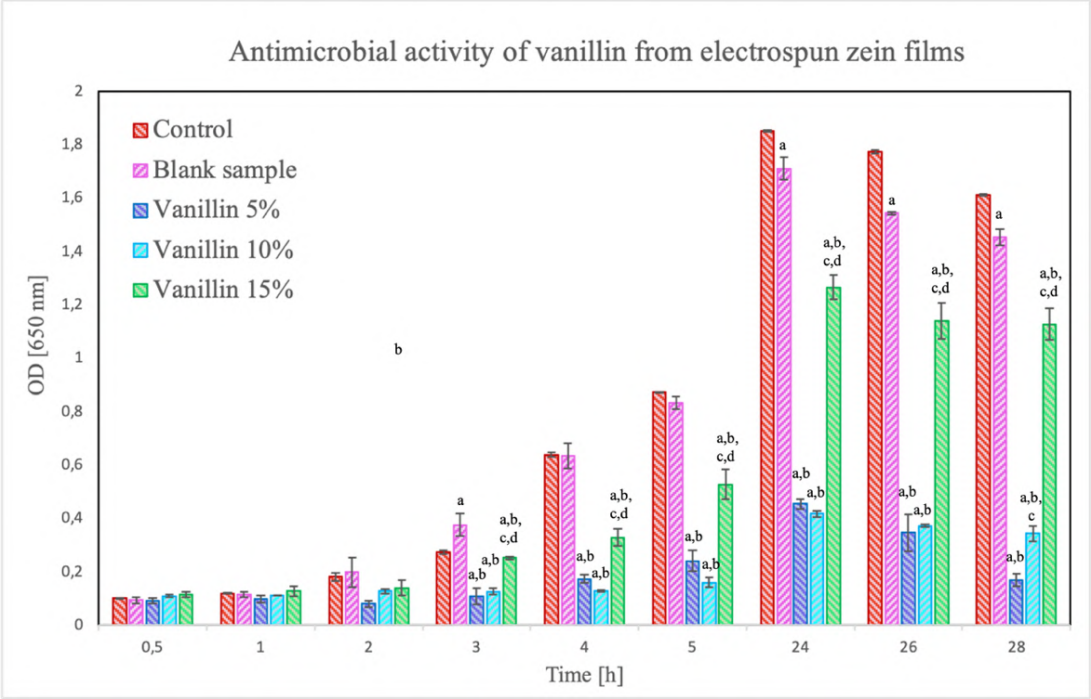


Figure 44: Antimicrobial activity of different vanillin loading into zein electrospun films against *Saccharomyces cerevisiae*. Different symbols indicate statistically significant differences among values over time ($p<0.05$) of dataset with different vanillin loading. a: statistically different from control sample; b statistically different from blank sample; c statistically different from vanillin 5%; d statistically different from vanillin 10%.

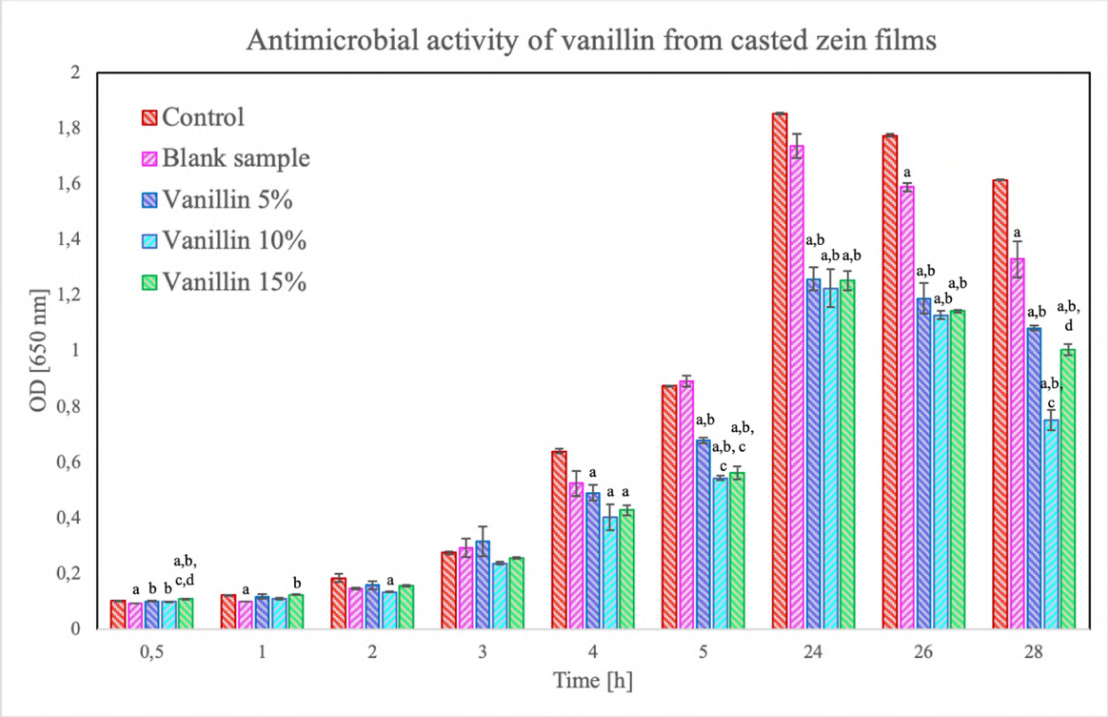


Figure 45: Antimicrobial activity of different vanillin loading into zein casted films against *Saccharomyces cerevisiae.* Different symbols indicate statistically significant differences among values over time ($p<0.05$) of dataset with different vanillin loading. a: statistically different from control sample; b statistically different from blank sample; c statistically different from vanillin 5%; d statistically different from vanillin 10%.

The results obtained showed that, with respect to the growth of the control sample (medium with yeast), the blank sample (unloaded zein) had a similar trend for both polymeric structures tested, highlighting that the zein has practically no effect on the yeast, i.e., it does not inhibit it but it does not even favor its growth as a substrate. On the other hand, in the case of the samples loaded with vanillin produced by electrospinning, yeast growth was inhibited by the presence of vanillin, ($p<0.05$), and the samples that showed the greatest antimicrobial activity against yeast were those produced with the lowest concentrations of vanillin, 5% and 10% w/w of vanillin with respect to weight of polymer. In the case of the samples produced by solvent casting all three concentrations of vanillin showed similar antimicrobial activity ($p<0.05$), slightly higher in the cases of loaded vanillin at 10% and 15% w/w. For these samples, observing the results obtained after 24 h, corresponding to the maximum growth phase reached, the reduction of yeast cells was found to be equal to about 75% for the electrospun samples by lowering the number of yeast cells from 15 x 10^6 cells per mL to about 3.5 x 10^6 and about 30% for solvent casting samples with a reduction in cell number to 10 x 10^6 cells per mL. This difference in behavior is due to the fact that the OD measurements can be affected by the different vanillin release capacities of the polymeric structures

produced. In particular, the electrospun films, having a greater surface exposed to the culture medium, are subject to a greater swelling phenomenon which involves a greater contact between the medium and the vanillin contained in the fibrous structure, as also observed during the migration tests in food simulants. Therefore, these interesting results, albeit conducted in a preliminary way, allowed to conclude that high concentrations of vanillin in the zein films are not necessary in order to exert an antimicrobial action against *Saccharomyces cerevisiae* and that the optimal concentration of vanillin to be loaded seems to be equal at 10% w/w with respect to zein, i.e. 6 mM, comparable to that observed by other authors who reported a concentration of pure vanillin, not dispersed in a polymeric matrix, equal to 5 mM to obtain an inhibition of about 50% while they reported a complete inhibition effect for values equal to 20 mM, not investigated in this work [246]. Other authors have observed that at concentrations of vanillin equal to 6 mM it is possible to obtain a reduction of 73% also against *E. coli* [237].

As an important conclusion for this section 2.2, it is important to underline how the methods used made it possible to obtain migration kinetics of the loaded active compound, very different from each other and dependent above all on the production technique of the supports as well as on the quantity of active compound loaded. This therefore entails the possibility of choosing which technique to adopt based on the choice of the desired final application, and in particular, based on the prediction of the shelf-life within which the antimicrobial compound has to act.

2.3 Loading of spent coffee grounds extract on different zein supports

The data that will be discussed in this section has been already published:

- M. Pettinato, E. Drago, R. Campardelli, P. Perego. Spent Coffee Grounds Extract for Active Food Packaging Production. *Chemical Engineering Transaction*, 2021, 87, 583-588. Doi: 10.3303/CET2187098.
- E. Drago, M. Pettinato, R. Campardelli, G. Firpo, E. Lertora, P. Perego. Zein and Spent Coffee Grounds Extract as a Green Combination for Sustainable Food Active Packaging Production: An Investigation on the Effects of the Production Processes. *Applied Science*, 2022, 12, 11311. Doi: 10.3390/app122211311.

In this section, zein-based polymeric films have been made enriched, instead of with a single molecule (vanillin), with a natural extract from spent coffee grounds, obtained through a non-conventional extraction technique, high pressures and temperatures extraction. In fact, as reported in the "Introduction", it seems that extracts composed of multiple active molecules are more efficient than a single molecule in terms of antimicrobial and antioxidant power. Electrospinning, solvent casting, and spin coating were employed to produce different polymer structures with tunable characteristics. Physical properties such as tensile strength, oxygen barrier, and release of bioactive compounds of the extract were investigated and it was found that they can be modified by varying the production technique and load of the extract, depending on the application desired. The materials thus developed seem to have the potential to offer a valid alternative to traditional plastic packaging, with beneficial effects on food and the environment. Furthermore, considering the global extension of the coffee wastes produced annually, their treatment for the recovery of substances with high-added value to be used in the packaging field offers a valid alternative to their conventional disposal.

2.3.1 Materials and Methods

2.3.1.1 Materials

Zein, glycerol, 2,2'-azino-bis (3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS), chlorogenic acid standard, ethanol, acetonitrile and methanol were purchased from Sigma-Aldrich (Milan, Italy). Caffeine standard was purchased from VWR Chemicals (Milan, Italy). Spent coffee grounds were recovered from vending-machine of the Department of Civil, Chemical and Environmental Engineering of the University of Genoa. Oxygen gas was used with purity grade N5.0.

2.3.1.2 Extract and polymeric solution preparation

The natural extract was produced starting from dried spent coffee grounds (SCG) of *Coffeea canephora* stored in the dark at room temperature, using a stainless-steel stirred extractor (Parr Instruments Company, model 350 M – 4650 Series, Illinois, USA) to performed an high pressure and temperature liquid extraction (HPTE), sketched in Figure 46, using ethanol 54% v/v as extracting solvent, a temperature and a pressure of 150 °C and 7.2 bar respectively for 1 h, following the protocol reported in Pettinato et al., 2020, in which all the characterizations carried out on the investigated extract are reported [247]. Furthermore, the extraction was conducted under inert atmosphere to reduce degradation reaction. The hydroalcoholic extract produced was loaded in liquid form into the zein solutions with a load varying between 0% and 45% w/w with respect to the weight of zein. The study was conducted by verifying that the hydroalcoholic solution used for the extraction, as solvent composition, was similar, albeit diluted, to that used to dissolve the polymer in order to avoid both the precipitation of the extract once added to the polymer and the reduction of zein solubility. In fact, the ratio between polymer and solvent, after the addition of the liquid extract, has been kept constant, in order not to change what was investigated in section 2.1 relating to solution properties. The polymeric solution was prepared according to the method already described with some modifications: the zein was dissolved in a hydroalcoholic solution of ethanol at 80% v/v and placed at 70 °C until the complete solubilization of the polymer. The solution was then cooled to 40 °C, temperature at which both the extract and the glycerol were added. Glycerol was used only in the solutions to be processed by solvent casting and spin coating due to the need of using a plasticizer.

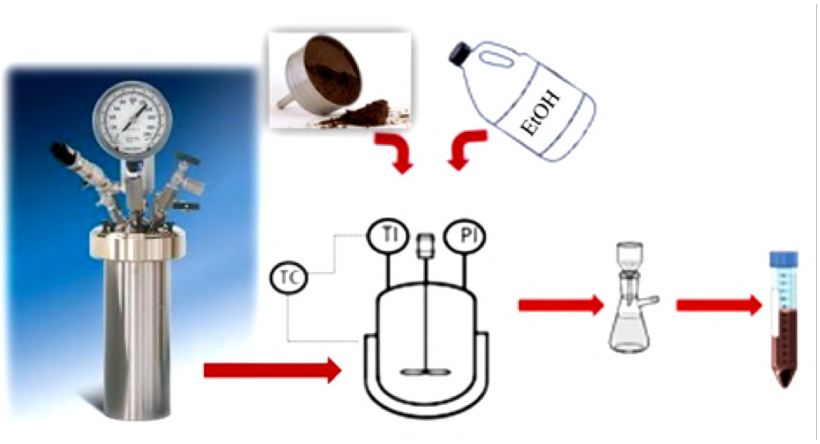


Figure 46: HPTE extractor and schematic representation of the SCG extract production.

2.3.1.3 Production of zein films loaded with SCG extract with different techniques

Zein-based films and zein loaded SCG films were produced by three different techniques: electrospinning, solvent casting, and spin coating for comparison purposes.

Electrospun films of zein at 35% w/w concentration, were obtained by feeding 6 mL of the hydroalcoholic polymeric solution prepared as previously reported, using a voltage of 17 kV, a flow rate of 1.2 mL/h and a needle-collector distance of 16 cm. After deposition, mats were left for 2 h in desiccator to ensure complete drying. The films were also produced by solvent casting process by pouring 2.5 mL of each solution composed of zein at 20% w/w concentration, both those with unloaded zein and those loaded with the different concentrations of extract. The samples were then dried in an oven at 60 °C for 2 h and removed after having cooled them in a desiccator. Finally, the solutions prepared for the solvent casting process were also processed by spin coating. In particular, 4 mL of each solution was poured onto Petri dishes placed on a rotating disc in a centrifuge (Spin 150 APT GmbH, Germany), of which a process schematization is shown in Figure 47. The film was obtained by spinning the disc at 100 rpm for 10 seconds. After deposition, the samples were placed in an oven at 60 °C for 2 h and cooled in a desiccator before the detachment. All the samples were produced in triplicate.

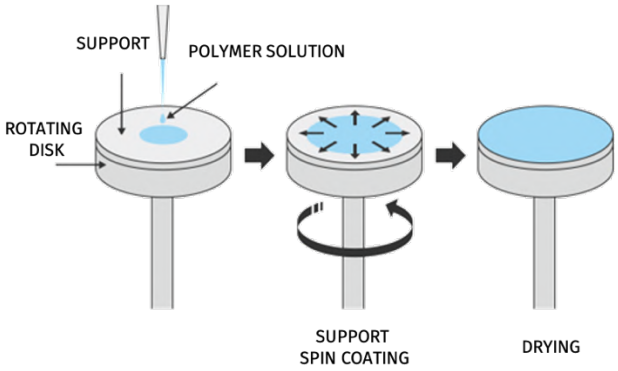


Figure 47: Scheme of the spin coating process.

2.3.1.4 Morphological characterization

The morphology analysis of the films obtained was performed by ultra-high resolution Field emission Scanning electron microscope (UHR-FE-SEM, CrossBeam 1540 XB, Zeiss GmbH, Germany). The thickness of the films produced was determined by optical microscopy (Olympus BX51) with at least five measurements for each sample. For electrospun films, the mean fibers diameter was determined by the ImageJ software (NIH, Bethesda, MD, US) analyzing at least 300 fibers per sample.

2.3.1.5 Release tests

To perform the release tests, the samples were trimmed to have 9 cm diameter specimens that were completely immersed in a food simulant, ethanol 10% v/v ethanol (20 mL). The release tests, unlike the migration tests, were conducted dynamically, placing the samples on an orbital shaker at 250 rpm at room temperature. Release liquid samples were collected at different time intervals, centrifuged at 35273 × g for 10 min at 4 °C (Alliance Bio Expertise, MF 20-R, France), and the caffeine concentration was analyzed by high-performance liquid chromatography (Agilent 1100 HPLC series, Palo Alto, CA), coupled to a diode array detector and equipped with a C18 reverse phase column (Vydac, Hesperia, CA). 100 µL of the centrifuged simulant supernatant was injected undiluted for each run. Mobile phases composed of water/acetic acid (99: 1% (v/v) (solvent A) and methanol/acetonitrile (50: 50%, v/v) (solvent B) were used and the solvent gradient was set as follows: 100% A for 5 min, 5% to 30% B in 25 min, 30% to 40% B in 10 min, 40% to 48% B in 5 min, 48% to 70% B in 10 min, 70% to 100% B in 5 min, isocratic at 100% B for 5 min, followed by return to initial conditions (10 min) and column equilibration (12 min). A flow rate of 1.0 mL/min at 30 °C was used.

The caffeine concentration in the samples was determined from the area under the caffeine peak in the chromatograms using the calibration curve obtained from standard caffeine solutions.

The radical scavenging capacity of the active molecules contained in thee SCG extract released from the polymeric support was determined by $ABTS^{\bullet+}$ assay and expressed as percentage inhibition according to the equation (6).

$$Inhibition\ (\%) = {(ABS_{blank} - ABS_{sample})}/{ABS_{blank}} \quad (6)$$

$ABTS^{\bullet+}$ working solution was prepared by adding $K_2S_2O_8$ to an aqueous solution of ABTS 7 mM up to a final concentration of 2.45 mM. Absorbances of the working solution (ABS_{blank}), and of 50 µL of sample mixed for 2 min with 1 mL of working solution (ABS_{sample}) were measured by an UV–vis spectrophotometer (Lambda 25, Perkin Elmer, Wellesley, MA, USA) at 734 nm.

2.3.1.6 Oxygen barrier properties

The permeability properties of the films produced in terms of permeability P to oxygen and diffusivity D, were analyzed using a homemade ultrahigh-vacuum equipment, showed in Figure 48, specifically modified for permeability measurements based on membrane process. This equipment incorporates innovative features compared to those available on the market, such as the residual gas analyzer (RGA100, Standford Research Systems) for the measurements of selected gases. Furthermore, it can work both in static and dynamic conditions i.e., with a constant-volume-variable-

pressure or constant-pressure respectively. Since the RGA sensitivity can be estimated with a not negligible uncertainty given by the calibration of the instrument, permeability is usually determined by the static method with more accuracy The procedure followed to provide the results was a manometric procedure, as defined in ASTM D1434-82 [248].

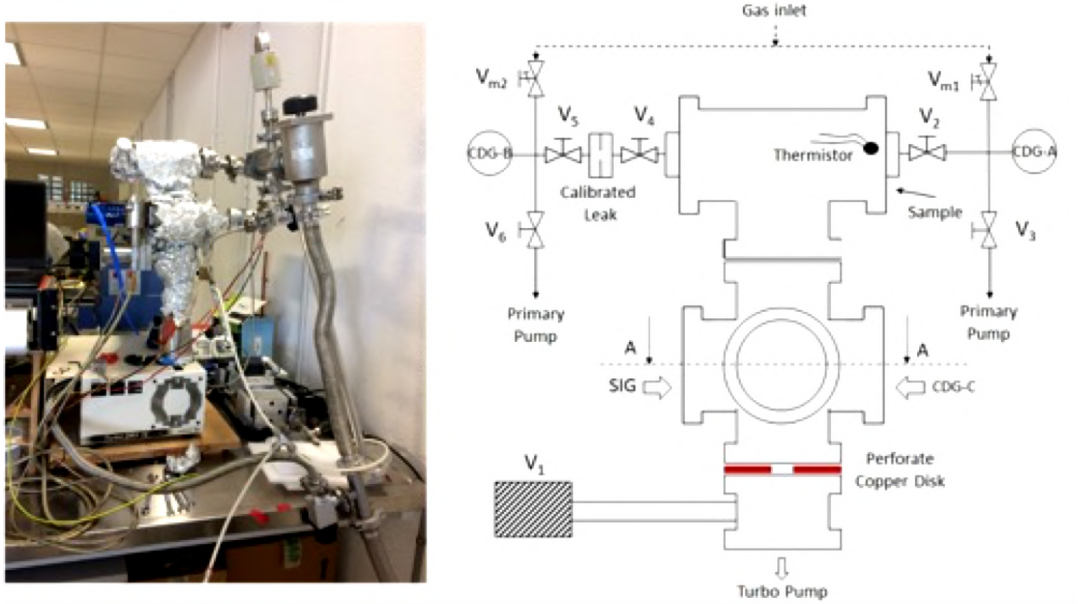


Figure 48: Ultra-high vacuum homemade instrument based on membrane processes for gas permeability measurements [210].

The samples are placed between two chambers, one upstream and one downstream. The downstream chamber works in an ultra-high vacuum (about 10^{-7}-10^{-12} Pa). All the samples, before the test, were characterized in terms of thickness in order to adjust the surface to be exposed to the test to obtain a mechanical stability at a differential pressure of 1 atm applied. In general, the diffusivity of the oxygen in the samples was calculated by the lag time method. Assuming to introduce the gas upstream of the sample at the instant t=0, it is possible to calculate the quantity of gas Q(t) that has passed through the film at the instant t > t*, where t* is the time in which it achieves the steady-state condition using equation (7):

$$Q(t) = A(\int_0^{t*} J(t)dt + \int_{t*}^{\tau} J_{SS}dt) \quad (7)$$

Where $J(t)$ is the gas transfer rate per unit area, J_{SS} is the transfer rate at the steady-state and τ is the total measurement time.

The diffusivity is then obtained by equation (8) [249]:

$$D = {L^2 J_{SS}}\Big/{6(J_{SS}t^* + \int_{t^*}^{\tau} J(t)dt)} \quad (8)$$

Where D is the diffusivity [cm^2/s] and L is the film thickness [cm].
Since the gas transfer rate per unit area of the film can be considered proportional to the ionic current measured by the RGA for the specific gas, equation (8) can be written as in equation (9):

$$D = {L^2 (I_{SS} - I_0)}\Big/{6 \int_0^{t^*} I_{SS} - I(t)dt} \quad (9)$$

Where I_{SS} is the ion current of the gas at the end of the measurement when steady-state condition is reached, and I_0 is the background ion current of the gas, i.e., just before the gas is introduced in the upstream chamber. The integral in equation (9) can be calculated numerically through the trapezoidal rule. Finally, the permeability P [cm^2/s] can be obtained by equation (10) which derives from Fick's law considering that the samples are thick enough to consider negligible the surface impedance respect to the bulk [210].

$$P = {JL}\Big/{\Delta p} \quad (10)$$

Where Δp that represents the difference between the upstream and the downstream pressures, causes the gas diffusion through the film layer, considered as an infinite plate.
The permeability and diffusivity were evaluated graphically as the slope and the intercept of the fitted line with the diffusion equation (9), obtained by the least square method. The background registered due to outgassing has to be subtracted from the curves obtained. Furthermore, the diffusivity using the static method, can be calculated as reported in equation (11) from the lag time t_L, extrapolated at a pressure equal to zero.

$$D = {L^2}\Big/{6t_L} \quad (11)$$

By calculating the diffusivity and the permeability, it was also possible calculate the solubility of the oxygen into the films, according to Henry's law reported in equation (12):

$$S = {P}\Big/{D} \quad (12)$$

The measurements were performed at room temperature (25 °C) on all spin coated samples at all concentrations of SCG extract.

2.3.1.7 Mechanical properties

The mechanical properties of the films were evaluated in terms of tensile strength (TS), elongation at break (EB) and Young's modulus (YM) using the Instron 8802 Machine according to ASTM638 standard method and UNI EN ISO 527 for polymeric materials [201]. The samples were cut into strips of 40 x 90 mm^2 and mounted between the grips. A 5 kN load cell and a cross-head speed of 1 mm/min were used for the tests. At least five specimens were tested for each measurement.

2.3.1.8 Statistical analysis

Statistica v8.0 software (StatSoft, Tulsa, OK, USA) was used to perform a statistical evaluation of experimental data. Analysis of variance (ANOVA) and Tukey's post-hoc test was carried out to assess the significance of differences among groups with statistical significance considered at a probability value (p) < 0.05.

2.3.2 Results and discussion

2.3.2.1 Spent coffee ground extract

The spent coffee grounds extract was chosen because the presence of numerous antioxidant compounds within it, that act as a scavenger of free radicals, can help slow down the degradation of foods by increasing their shelf-life. In particular, among these compounds, the mains are caffeine, chlorogenic acid, followed by protocatechuic acid, ferulic acids and melanoidins. The SCG extract produced using the high pressure and temperature extraction (HPTE) performed at 150 °C in ethanol 54% v/v, was analyzed by HPLC. The chromatogram obtained is shown in the Figure 49, from which it can be seen that the main peak is that of caffeine (1.032 ± 0.018 mg/mL) with a retention time of 23.7 min. Therefore, caffeine was chosen as the reference compound to be quantified through the release tests, the results of which will be shown below. Instead, the overall antioxidant action of the extract, in terms of total polyphenol content, was assessed by an ABTS test which revealed a total polyphenol content equal to 4.96 ± 0.25 mg $_{\text{caffeic acid equivalents}}$/mL, with an antiradical power of 46.2 ± 5.2 μg_{TE}/L of extract.

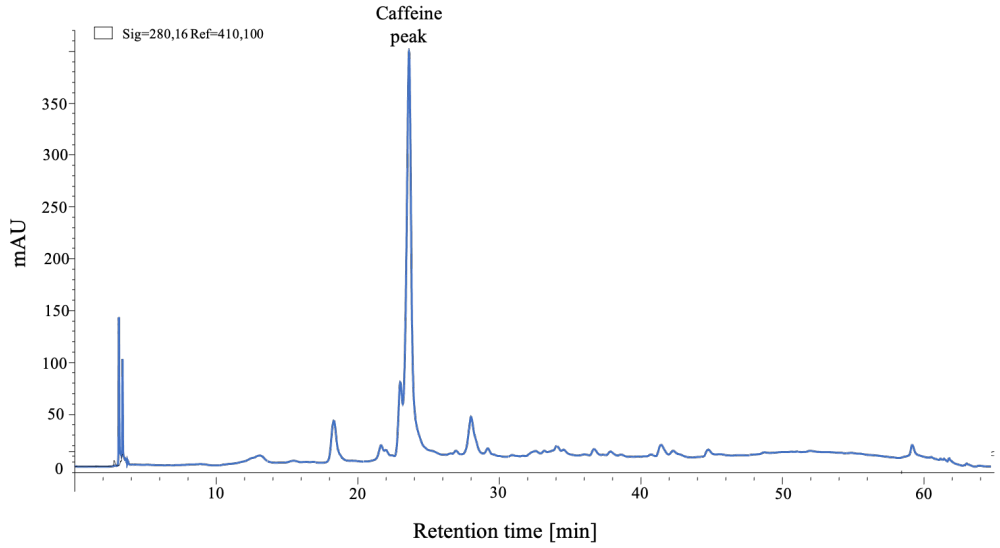


Figure 49: Spent coffee grounds extract chromatogram obtained via HPLC.

2.3.2.2 Zein enriched with SCG extract film production

Given the temperature sensitivity of the antioxidants, the processes selected for their incorporation into polymeric films were electrospinning, solvent casting and spin coating, which allow to work in mild conditions. All zein samples were produced by varying the concentration of extract from 0%, produced as control samples, to 15%, 20%, 35% and 45% w/w of extract with respect to the weight of zein.

The techniques used for the production of the films have made it possible to obtain, as in the case of zein loaded with vanillin, different polymeric structures useful for conducting a comparison in terms of physico-chemical properties. In particular, as shown in Figure 50, opaque, porous films were obtained by means of electrospinning consisting of an intertwining of fibers arranged randomly that give the film a neutral color, tending to white, independent of the color of the processed zein which, by nature, it has an intense yellow color. Instead, through solvent casting and spin coating, it was possible to obtain transparent, dense films, with a strong yellow color that increased its intensity as the presence of SCG extract increased, which given its source of origin, presents a deep brown color. Furthermore, the loaded extract seems to have perfectly mixed with the polymer and distributed evenly in the polymer films, not having noticed any agglomerates.

It should be emphasized that the spin coating technique is usually used as a coating technique for various types of supports and not as a production process of the support itself. In this case, having noticed in the previous chapter dedicated to the solvent casting of zein loaded with vanillin, that the thickness of these films was rather inhomogeneous, it was decided to exploit the centrifugal force of the rotating support provided in the spin coating technique, to standardize the deposition of the zein solution on the Petri dishes. To do this, the Petri dishes were used in reverse, i.e., without edges that

hinder the distribution of the solution during rotation. Therefore, the spin coating technique was used to investigate a possible improvement in the uniformity of the films.

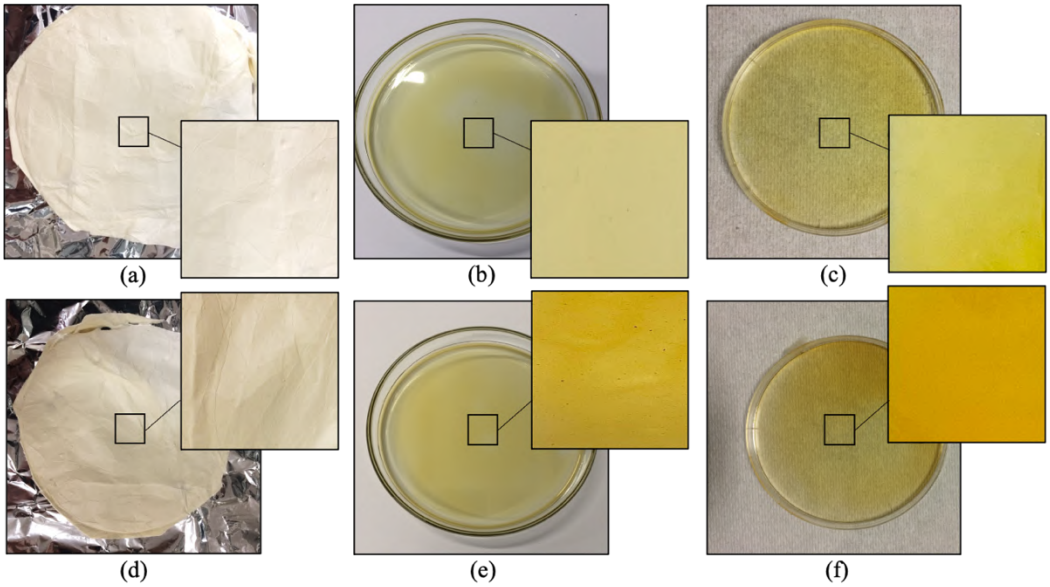


Figure 50: Unloaded zein films obtained through (a) electrospinning, (b) solvent casting, and (c) spin coating, SCG 45% w/w loaded zein films obtained through (d) electrospinning, (e) solvent casting and (f) spin coating with enlargement for highlighting the color variation.

2.3.2.3 Morphological characterization

To confirm the success of the tests and, specifically, the difference between solvent casting and spin coating, the microscopic structure of the samples was analyzed by SEM. In Figure 51, are reported an image for each production technique, related to the control sample of unloaded zein.

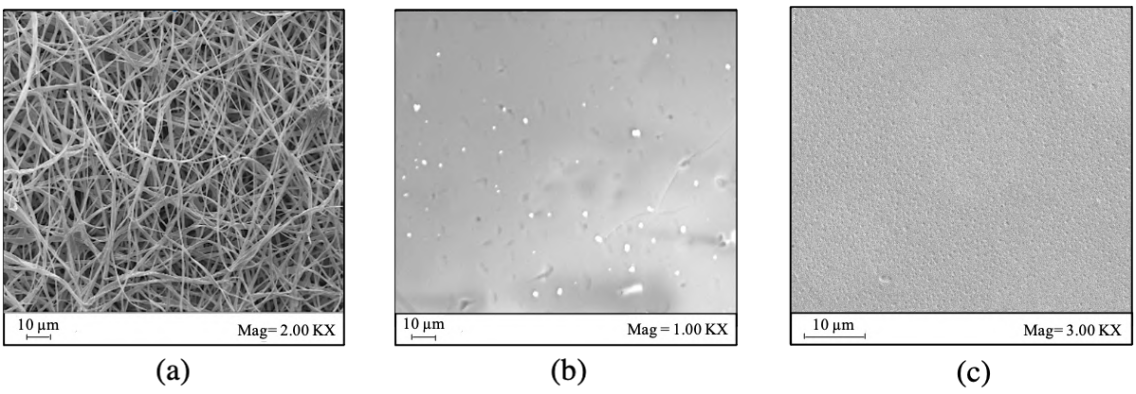


Figure 51: SEM image of (a) electrospun zein film; (b) solvent casting zein film; (c) spin coating zein film.

The difference between the structures produced by electrospinning and those produced by casting has already been commented on previously. In this case it is interesting to note the difference between the samples produced between solvent casting (Figure 51b) and those produced by spin coating (Figure 51c). In fact, the surface of the latter was much more wrinkled and uniform than the others which instead have scattered defects. This difference is probably attributable to the rotation of the supports which, in addition to acting by evenly distributing the volumes poured, also acts on the arrangement of the polymer chains and glycerol.

Table 17 shows the results obtained in terms of film thickness and grammage (specific weight), depending on the concentration of SCG extract loaded for each technique, to investigate whether the addition of the extract has any effect on these properties. The results are expressed as mean value ± standard deviation.

Table 17: Thickness (σ) and grammage of zein samples produced by electrospinning, solvent casting and spin coating by varying the SCG extract concentration.

SCG extract % w/w	ELECTROSPINNING		SOLVENT CASTING		SPIN COATING	
	σ (μm)	Grammage (g/m^2)	σ (μm)	Grammage (g/m^2)	σ (μm)	Grammage (g/m^2)
0	54.0±7.5	33.37±0.6	51.23±9.4	73.23±1.5	55.80±3.2	61.22±0.9
15	35.8±2.9	35.12±10.2	37.27±7.2	76.34±6.3	53.50±3.1	62.86±2.8
20	27.4±6.4	26.50±3.8	37.93±9.4	80.62±1.0	54.25±2.9	63.26±2.5
35	31.2±4.4	23.92±4.3	43.57±11.4	93.29±0.9	50.75±1.5	46.24±1.0
45	14.4±3.3	19.47±3.3	50.80±11.4	66.96±5.0	51.28±3.3	46.29±0.5

As expected, the electrospun films showed a lower specific weight than the other techniques being porous materials. Furthermore, the thickness of the electrospun films proved to be particularly inhomogeneous, as also in the case of the zein films loaded with vanillin, mainly due to the deposition of the fibers on the collector which, occurring randomly, cannot ensure a uniform thickness unless it is processed much more material than the 6 mL provided here. The concentration of the extract also seems to influence the thickness of the electrospun films, in fact, as can be seen from the SEM images

depicting the fibrous structures of the samples and the distribution of the diameters, shown in Figure 52, the extract has an evident influence on the diameter of the fibers. In particular, as the concentration of the extract increases, the average diameter of the fibers.

Furthermore, it is also possible to observe a change in the shape of the fibers which, compared to the ribbon-like structure of the control sample of unloaded zein, have assumed a more filiform shape with increasing concentration of the extract.

No remarkable effect at the variation of SCG loading was noticed for solvent casting and spin coating samples. The spin coating appeared to be more reproducible in terms of film thickness and specific weight, demonstrating the smoothing effect provided by the rotation of the support which had been hypothesized as a means to improve the solvent casting.

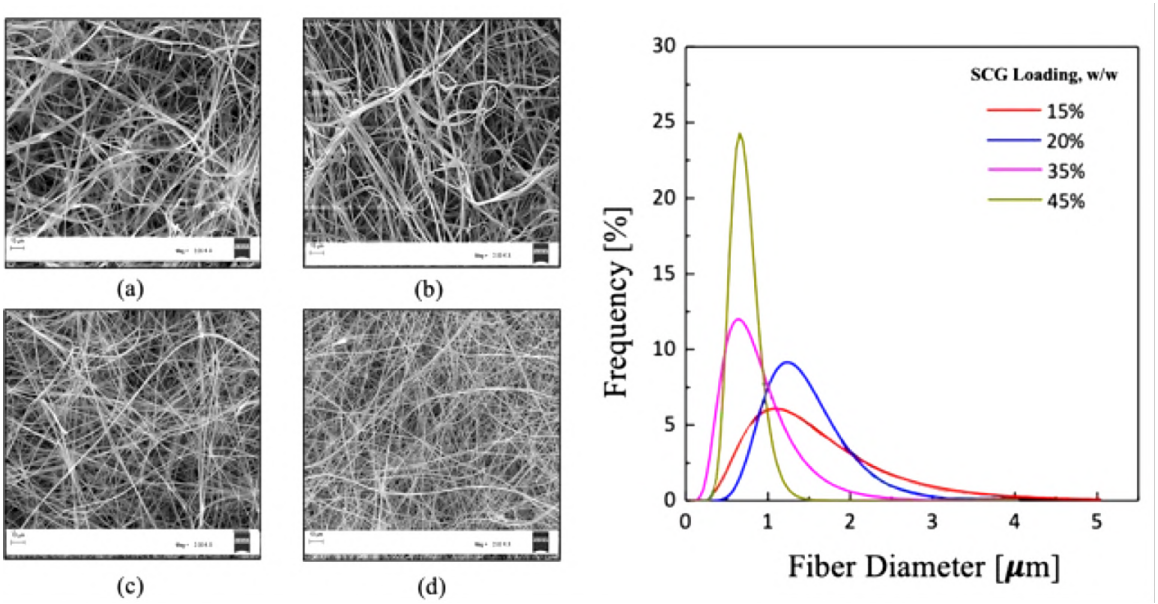


Figure 52: SEM images of electrospun zein fibers loaded with (a) 15% w/w, (b) 20% w/w, (c) 35% w/w, (d) 45% w/w SCG extract and fibers diameter distribution.

2.3.2.4 Release tests in food simulant

The dynamic release was carried out by immersion tests in 10% v/v ethanol as food simulant at room temperature (25 °C). These tests had the aim of evaluating the release over time of caffeine, identified as the greatest exponent of the extract, from the zein films produced using the three techniques to conduct a comparison. In addition, to evaluate the overall anti-radical power of the extract, colorimetric tests were conducted using an ABTS assay for the evaluation of inhibition.

The results of the release tests from the films produced using the three techniques are shown in Figure 53 and expressed as mg of caffein migrated per kg of zein film as requested from EU Reg. 1416/2016 for specific migration. Starting from the samples produced by electrospinning, the results of the caffeine release tests reported in Figure 53a, shows that, the fibrous structure of these samples, thanks to the large surface exposed to the simulant and therefore to mass transfer phenomena, as it had been observed for vanillin, it facilitates the release of loaded compounds. In fact, the plateau is reached very quickly for all the concentrations of extract analyzed.

Figure 53b and Figure 53c show the caffeine releases from the samples produced by solvent casting and spin coating respectively. In the case of solvent casting, it was possible to observe a slowly increasing release over the time, without reaching a plateau in the 48 h of test conducted. Furthermore, unlike the electrospun samples, it was possible to observe a clear dependence on the concentration of the loaded SCG extract in the polymer. On the other hand, in the case of the samples produced by spin coating, the caffeine releases proved to be faster than those of the previous case, especially in the first hours of testing. Furthermore, the samples produced by loading 20% w/w of extract showed a release higher than the other samples, equal to that of the case at 45% w/w of SCG extract. Probably, the different behavior observed is due to the centrifugal force impressed on the solution during the process. The rotation of the support may in fact have caused the extract to be distributed superficially, resulting in a faster release than the other samples.

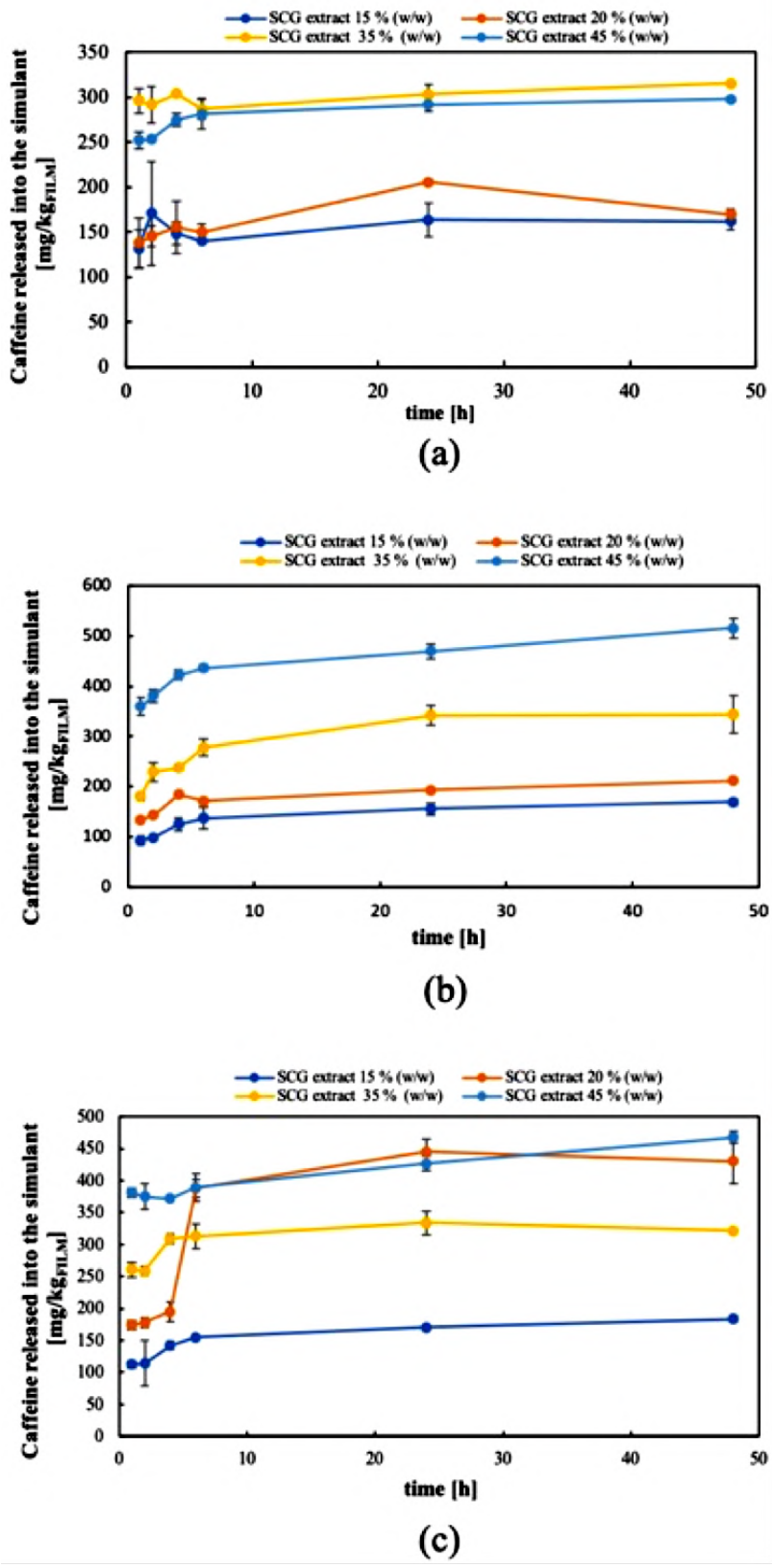


Figure 53: Results of caffeine release tests in ethanol 10% v/v from films obtained by (a) electrospinning, (b) solvent casting and (c) spin coating.

As concerns the antiradical power of the extract, Figure 54 reports the radical $ABTS^{\bullet+}$ inhibition exerted by aliquots of the food simulant sampled over time. The antiradical power evaluated by the ABTS assay is not related to the concentration of caffeine, as its specific antioxidant activity cannot be detected with the colorimetric method used, but to the other antioxidant compounds, mainly polyphenols and melanoidins. From Figure 54a, which shows the results of the ABTS assay for the evaluation of inhibition in electrospun samples, it was possible to observe that in addition to a rapid release of caffeine over time, another phenomenon also occurs, namely the rapid degradation of the extract over time with consequent loss of inhibition capacity. In Figure 54b, relating to the samples produced by solvent casting, it was found that, at a certain fixed time, the antiradical power increased as the extract load increased. Furthermore, in the first 24 h the inhibition values of the samples loaded with less extract remained practically constant, with an increase only after 24 h. While those with the highest concentration of extract maintained a constant inhibition value after the first 2 hours of the test.

Finally, in the case of spin coating, the rapid release of caffeine from this type of samples was also confirmed by the greater inhibition effect observed in the first hours of the test. In fact, with the exception of the samples with the lowest concentration of extract, for which the inhibition remained around values just above 10%, in the other cases the inhibition values were much higher than the other techniques in the first 4 hours of tests, and then decrease. Also in this case, as for electrospinning, it is possible that such a rapid initial release involves the exposure of the extract to degradation reactions with consequent loss of antiradical power.

Furthermore, the different distribution of loaded extract into spin coated sample can have altered the structure of the samples and the release of the compounds. The different behavior observed between casted and spin coated samples was also observed by López-Rubio et al., 2020, who compared the two fabrication processes in the production of amaranth protein isolate-based films, finding a strong dependance of the film's physical properties on the production process [250].

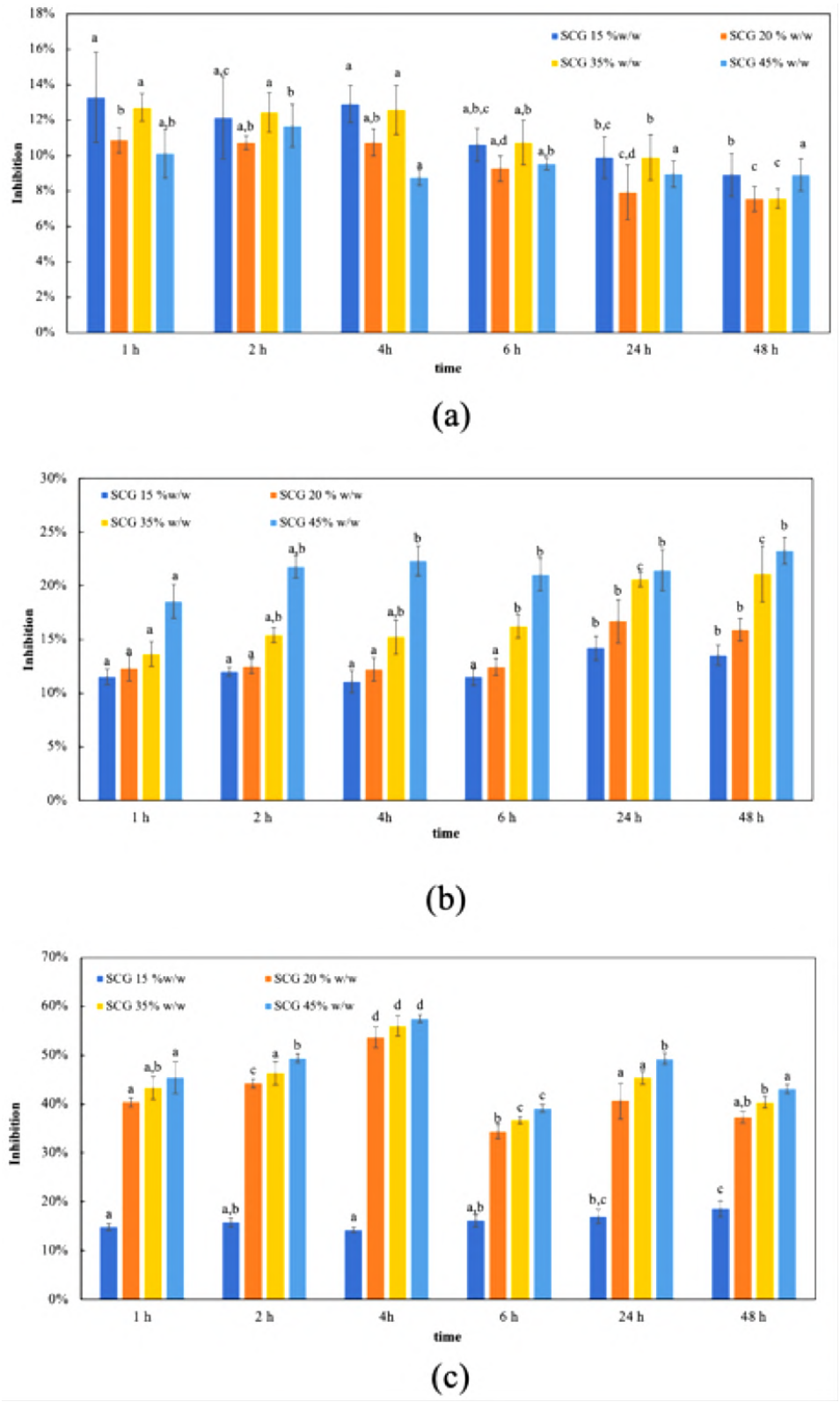


Figure 54:Results of radical ABTS$^{\bullet+}$ inhibition for (a) electrospinning, (b) solvent casting and (c) spin coating. Different symbols indicate statistically significant differences among values over time ($p<0.05$) of dataset with the same extract loading.

The correlation between the concentration of caffeine released and inhibition power has been shown to be linear only in the case of solvent casting, as can be seen from Figure 55, demonstrating that caffeine can be used as a marker for the entire behavior of the extract regarding migration to the selected simulant. On the other hand, for the other two techniques a linear correlation was not found, indicating the occurrence of activity losses of the antioxidants.

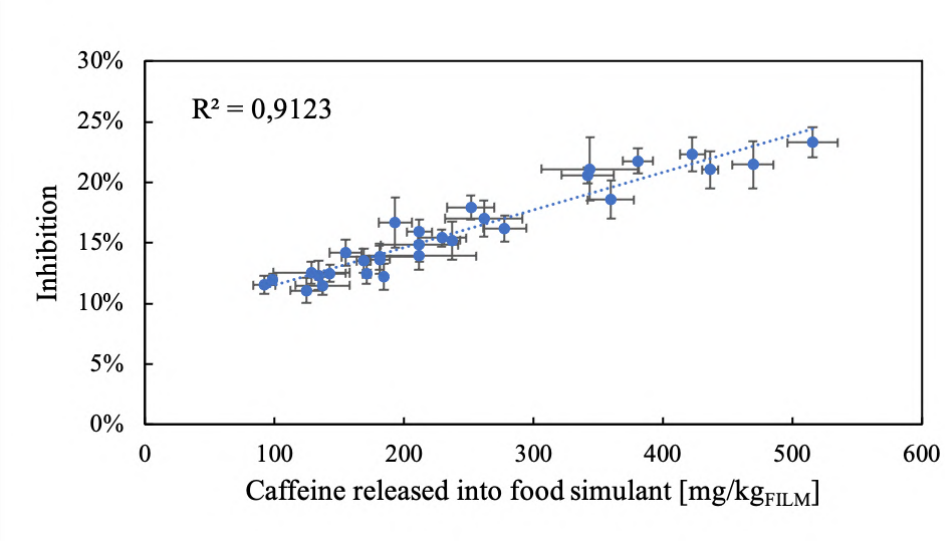


Figure 55: Linear correlation between the caffeine concentration released and radical inhibition power of the extract migrated into the food simulant from solvent casting.

2.3.2.5 Oxygen barrier properties

The analyzes relating to oxygen permeability were conducted by the measurements of oxygen permeability, diffusivity and solubility applying 1 atm of differential pressure across the zein films in a known volume. As reported in the "Materials and Methods" of this section, the oxygen permeability was obtained through the static method considering the slope of the curve pressure-time at the steady state fitted with the diffusion equation by the least square method, as represented in Figure 56 for the blank sample (unloaded zein).

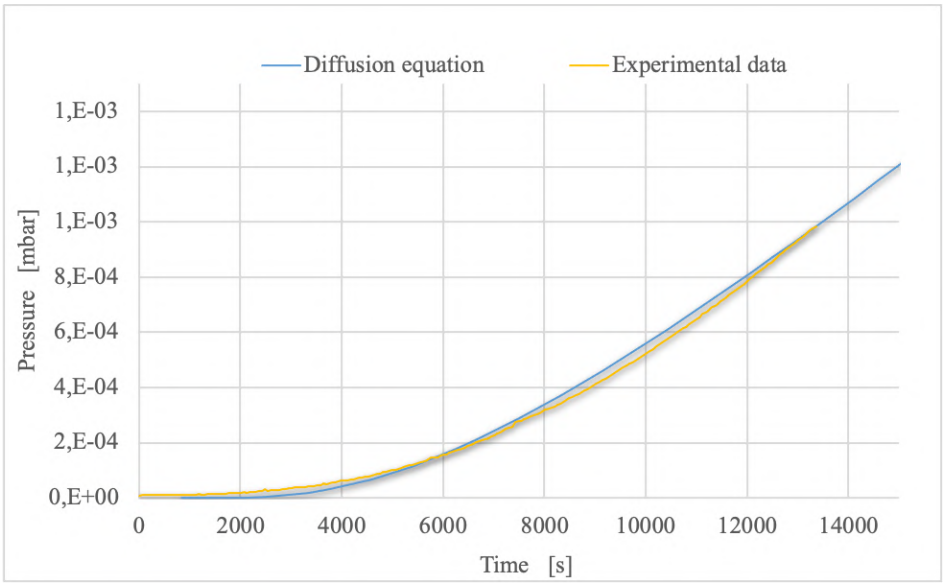


Figure 56: Fitting with the diffusion equation of the experimental data obtained by the blank sample using the static method for oxygen.

Table 18 shows the results relating to the samples produced by spin coating, as the other samples were not suitable for measurements. In particular, the electrospun zein films, being porous materials, were found to be completely transparent to the gas flow, as expected. The samples produced by solvent casting, on the other hand, were measurable but affected by the non-uniformity of thickness, which is why it was initially decided to try to use the spin coating technique.

Table 18: Oxygen permeability, diffusivity and solubility measurements results for spin coating films at different SCG concentration, from 0% to 45% w/w.

Sample	**Permeability [cm^2/s]**	**Diffusivity [cm^2/s]**	**Solubility [-]**
SCG 0%	4.65E-09 ± 4.2E-10	6.00E-10 ± 7.2E-11	7.75 ± 5.81
SCG 15%	2.03E-10 ± 1.8E-11	9.36E-11 ± 1.1E-11	2.17 ± 1.63
SCG 20%	5.79E-11 ± 5.2E-12	1.27E-11 ± 1.5E-12	4.55 ± 3.42
SCG 35%	1.37E-11 ± 1.2E-12	8.33E-11 ± 1.0E-11	0.16 ± 0.12
SCG 45%	4.86E-11 ±4.3E-12	8.33E-11 ± 1.0E-11	0.58 ± 0.44

It is necessary to consider that, the uncertainty in permeability depends on the errors in membrane thickness and area, on the volume of the downstream chamber, and on the slope of the curve pressure-time at steady state. The uncertainty in diffusivity depends on the errors in the intercept and in the

thickness. For the setup used in this work, the relative uncertainty considering the uncertainties of all the individual contributions (i.e., pumping speed, RGA sensitivity, sample area, thickness, pressure difference, etc.) was evaluated to be of the order of 9% for permeability measurements and 12% for diffusivity measurements [210].

The value of oxygen permeability for unloaded zein samples agrees with literature results [251]. As expected, the values of these parameters decrease increasing the concentration of SCG extract, consequently increasing the oxygen barrier. However, the trend changes increasing quantity of SCG over 35% w/w, because above this value, it is possible that the quantity of SCG extract generates a swelling phenomenon of polymer increasing gas diffusion.

Comparing these permeability data with those of some plastics used in food packaging, as low-density polyethylene (LDPE) or Polyethylene terephthalate (PET), the spin coating films can provide an equivalent, or also increased oxygen barrier [211].

Considering the experimental procedure adopted, data reported are free from artefact respect to the physical aging of zein [251]. In fact, zein, as all polymers, suffers of physical aging depending also on the quantities of adsorbed gas, in particular for condensable gas as oxygen that can induce plasticization. Plasticization is based upon molecular chain reorganization of a polymer induced by high sorption of a condensable gas [252]. This phenomenon ensuing instability of the permeation properties over time.

2.3.2.6 Mechanical properties

The mechanical properties of the zein films were estimated through the evaluation of tensile strength (σ_M), elongation at break (ε_B) and Young's modulus (E).

The samples produced by electrospinning, if on the one hand they showed a unique and interesting behavior due to the ability to release compounds with high-added value in a short time, on the other hand they were not suitable for characterization in terms of mechanical properties, in addition to have been found to be completely permeable to oxygen, as described in the previous paragraph.

In fact, the electrospun samples showed evident brittleness in handling, a fact confirmed by other authors [253]. As this phase of the work is a feasibility study of the loading of active compounds and their effect on different supports, the electrospun samples were not subjected to mechanical tests, but possible solutions were identified to make them more manageable and to increase the evident lack of mechanical properties, that is to process more volume of solution, compared to the approximately 6 mL used in this work, which proved to be too few, and to use a polymeric blend to improve the properties of the zein.

Instead, the samples produced by the other two techniques were compared. First of all, the control samples, i.e., those without the presence of extract, were compared to evaluate the mechanical

characteristics only on the basis of the method of production and to assess whether the minimum tensile strength requirement, set at 3.5 MPa, required for packaging materials was satisfied [254]. From this study it emerged that only the samples produced by solvent casting satisfy this limit having an average value of tensile strength equal to 13.04 ± 3.10 MPa.

On the other hand, the samples produced by spin coating showed not to satisfy the limits presenting an average value of tensile strength equal to 1.13 ± 0.18 MPa. These results are clearly different from those of the samples obtained by casting and it could be due to the effect of the centrifugal force imparted by the rotation of the spin coater support on the arrangement of both polymer and plasticizer molecules. Having therefore identified solvent casting as the best method to produce films with a high tensile strength, mechanical tests were performed on these, as the concentration of SCG extract varied. The results are shown in Table 19 and are expressed as mean value ± standard deviation.

The results are to be considered satisfactory as regards the tensile strength, in fact, this property was found to be higher than those reported in other works and higher than the limit set for packaging materials equal to 3.5 MPa for all the samples. On the other hand, all the films presented poor elongation at break and high Young's modulus confirming the stiffness of the samples.

It is interesting to note that the presence of the SCG extract did not particularly affect the results neither in positive nor especially in negative, considering the addition of discontinuities to the film. Only in the case of the extract loaded at 45% w/w it was possible to notice a small increase in both tensile strength and stiffness, expressed by the Young's modulus and a small decrease in the elongation at break and, therefore, an increase in brittleness, but no statistically significant difference was highlighted by the ANOVA analysis, i.e. the extract does not influence the mechanical properties of the zein films.

Table 19: Mechanical properties of zein samples produced by solvent casting in terms of tensile strength (σ_M), elongation at break (ε_B) and Young's modulus (E). No statistically significant differences among values ($p>0.05$) were observed as the SCG extract percentage varied.

SCG extract %, w/w	**σ_M [MPa]**	**ε_B [%]**	**E [MPa]**
0	13.04 ± 3.10	0.63 ± 0.13	1860.12 ± 368.22
15	10.64 ± 2.06	0.92 ± 0.32	1403.52 ± 456.16
20	14.09 ± 1.76	0.59 ± 0.12	1846.83 ± 237.96
35	13.66 ± 1.61	0.76 ± 0.20	1895.32 ± 168.18
45	14.21 ± 3.99	0.58 ± 0.15	2128.25 ± 661.24

This brittleness observed, is known to be the greatest intrinsic weakness in vegetable proteins, but it could be also attributed to the amount of glycerol used. In fact, it has been studied by Zhang et al., 2015, that glycerol can act both as an anti-plasticizer, worsening the mechanical properties and as a plasticizer, improving them depending on the quantity used [255]. Even if the requirement for packaging materials is expressed in terms of tensile strength, the other mechanical characteristics such as the modulus of elasticity were not satisfactory, therefore it was decided to try to improve them by producing a polymeric blend through solvent casting as described in next chapter.

At the end of this section 2.3, it can be concluded that this part of the work made it possible to identify the potentials and limitations of three different polymer film production techniques. The materials produced using natural polymer, antioxidants from agri-food waste and green solvents have demonstrated their potential but also the limitations in their us as alternative materials to non-degradable plastics and synthetic additives. Finally, this study has shown how by varying the production process, starting from the same raw materials, the properties of the product can be drastically modified and adapted to the specific intended application.

3 DEVELOPMENT OF POLYMERIC BLEND BY SOLVENT CASTING

PURPOSE

This section had the aim of investigating the possible creation of a biopolymeric blend in order to improve the mechanical performances of the zein films produced by solvent casting. Unlike the electrospun films which, given the high release kinetics, the poor barrier properties, and the opacity were more suitable for a hypothetical application as active patches to be inserted inside the primary packaging, the films produced by solvent casting showed potential as a primary packaging material. Like most biopolymers, however, the mechanical properties of zein are rather poor, not so much in terms of tensile strength but rather in terms of elongation at break and deformation, as reported in the previous section. Therefore, in this chapter, zein has been blended together with another biopolymer with known film-forming properties, chitosan, chosen for its source of origin, its intrinsic antimicrobial properties, and its hydrophilic properties. Once the composition of the solution and the steps of the solvent casting process were optimized, the samples obtained were characterized in terms of mechanical properties and surface properties through tensile tests and contact angle measurements respectively. The results obtained have shown the possibility of obtaining transparent films with a better mechanical resistance than the single polymer. Despite the hydrophobic nature of zein, it was found that to reduce its hydrophobicity as well as to mix it with a more hydrophilic polymer, it is sufficient to choose the solvent and the film production methods correctly. Furthermore, it was possible to obtain films using a much lower quantity of polymers than that used for the film with zein alone which, from an economic point of view, could represent a considerable saving of raw materials, making these materials competitive with respect to plastics.

3.1. Materials and Methods

3.1.1 Polymeric blend formulation

The zein solutions (Sigma-Aldrich, Milan, Italy) were prepared by testing different concentrations of polymer starting from a concentration equal to 20% w/w dissolved in a hydroalcoholic solution of ethanol at 80% v/v (Carlo Erba Reagents, Milan, Italy), such as those treated in the previous paragraphs, up to solutions of 2% by weight of zein with respect to the hydroalcoholic solution of ethanol at 90% v/v following the suggestions reported in Zhang et al., 2019 [256]. The chitosan solutions (medium molecular weight, Sigma Aldrich, Milan, Italy) were prepared by dissolving

different polymer concentrations equal to 1%, 1.5%, 2%, 3%, and 4% w/v in a solution of acetic acid (Carlo Erba Reagents, Milan, Italy) 10% v/v in distilled water. The polymeric solutions were individually stirred at 80 °C for at least 1 h and were subsequently combined by varying the zein/chitosan ratio (10:1, 5:1, 2:1, 1:1, 1:2 w/w) in order to investigate which ratio did not cause the precipitation of chitosan, being insoluble in ethanol. The resulting solutions were stirred for an additional hour at 80 °C, then centrifuged at 35273 × g for 10 minutes at 4°C (Alliance Bio Expertise, MF 20-R, France) to remove impurities present in the chitosan, used as received from the supplier without further purification steps. The solutions were then poured (5 mL) onto glass Petri dishes. The samples were dried in an oven at 60 °C for a day and then left in the desiccator for another day before being detached. Glycerol (> 99.5%, liquid, Sigma-Aldrich, Milan, Italy), which in the previous chapters had been used to facilitate the removal of zein films, was used both by adding it to the zein solutions in a fixed percentage equal to 50% w/w with respect to the content of zein, either by spreading it only on the Petri dishes without adding it to the solutions. Samples of only zein and only chitosan, were also prepared following the procedures described above. All tests were conducted at least 7 times.

3.1.2 Morphological characterization

The samples obtained were analyzed by ultra-high resolution Field emission Scanning electron microscope (UHR-FE-SEM, CrossBeam 1540 XB, Zeiss GmbH, Germany).
The thicknesses of the films were evaluated through the use of a digital micrometer (3791G 0-150, Messzeuge, Germany) recording at least 6 measurements per sample.

3.1.3 Mechanical properties

Mechanical tests were conducted on all the samples produced using the Zwick Roell Z0.5 equipment in combination with Zwick Roell's testXpert III software. To perform the tensile tests, six specimens of size 1x5 cm^2 were made from each sample. A tensile speed of 10 mm/min, an initial grip separation of 21 mm and 500 N load cells were used for the analysis.

3.1.4 Wettability

The surface properties in terms of wettability were analyzed using the sessile drop method by measuring the contact angle (CA) using an equipment with a goniometer integrated and a video camera. The measurements were performed at room temperature on the samples of zein, chitosan and the polymer blend using a drop (about 5 μL) of distilled water as reference liquid. The CA was measured by recording the drop shape at instant zero and after 60 s to be sure that a state of

equilibrium was reached. At least 5 measurements were conducted for each sample. The results were expressed as the mean value of the contact angle ± standard deviation.

3.2 Results and Discussion

3.2.1 Polymeric blend optimization

The best conditions for obtaining zein-only and chitosan-only films were initially investigated using the solvent casting technique. As regards zein, the conditions identified in paragraph 2.1 were confirmed as the best for the production of transparent films without defects, i.e., a concentration of zein equal to 20% w/w compared to the solvent consisting of a hydroalcoholic mixture of ethanol with 80% v/v using glycerol as a plasticizer. The zein solutions were processed by pouring 2.5 mL on 9 cm diameter Petri dishes placed in an oven at 60 °C for 2 h before being detached. Lower concentrations of zein led to the formation of films too thin to be handled and analyzed.

For the chitosan, the solutions were prepared by dissolving different quantities of polymer, 1%, 1.5%, 2%, 3%, and 4% w/v in the solvent, an aqueous solution of acetic acid at 10% v/v. The 4% solution of chitosan was too viscous to be processed allowing to identify the concentration limit for this polymer. The other less concentrated solutions were processed by pouring 5 mL and leaving the samples in an oven at 60 °C for 24 h to obtain complete evaporation of the solvent. The 1% and 1.5% w/v chitosan films turned out to be too thin to be removed from the supports uniformly, while those at 2% and 3% led to the formation of transparent and manageable films. At this point, the work was focused on the production of the polymer blend starting from the union of the polymers at the concentrations identified as optimal respectively. Working with the solutions of zein at 20% w/w and chitosan at 2% w/v prepared in the respective solvents and mixed in a 1:1 ratio, it was not possible to obtain the complete mixing of the two solutions, in fact, the precipitation of the chitosan was noted with consequent separation of the two polymers once dried. Therefore, having the chitosan concentration limit, linked to viscosity, the concentration of zein was lowered. The best ratio of zein/chitosan polymers was found to be 1: 2. By keeping this ratio constant, the concentration of the individual polymers was increased to identify the optimal conditions of the blend, that was, zein at 2% and chitosan at 4%, pouring 5 mL of the resulting solution considering that, being very diluted, the zein decreases the viscosity of the chitosan solution, making it processable even at higher concentrations. A summary of the experimental tests carried out and the macroscopic results are shown in the Table 20. Furthermore, the best results were obtained by spreading a layer of glycerol directly on the Petri dishes rather than adding it to the solution using a hydroalcoholic solution of 90% ethanol for the zein instead of 80% v/v, thereby reducing the volume of water in the resulting solution.

Table 20: Set of experimental tests conducted for the production of a polymeric blend of zein and chitosan by solvent casting.

Zein [w/v]	Chitosan [w/v]	Results
20%	-	Good
10%	-	Too thin
-	4%	Too viscous
-	3%	Good
-	2%	Good
-	1.5%	Too thin
-	1%	Too thin
20%	2%	Not blended
10%	2%	Not blended
4%	2%	Not film formation
2%	2%	Film with surface defects
1%	2%	Too thin
1.5%	3%	Too thin
2%	4%	Good

3.2.2 Morphological characterization

Some examples of the samples produced and the relative morphology obtained by Field emission Scanning electron microscope are reported in Figure 57.

In particular, the chitosan films presented a very smooth and uniform surface (Figure 57b and Figure 57e), while the polymeric blend presented a rough surface with a uniform distribution of particles (Figure 57f) probably caused by the entanglement of the polymer chains of the two polymers, as also observed by Zhang et. al., 2019 [256]. Furthermore, a shrinkage of the polymeric blend was observed (Figure 57c) once it was detached from the supports, probably due to a necking of the polymer chains induced by the cooling from the 60 °C, used during the process, to the room temperature.

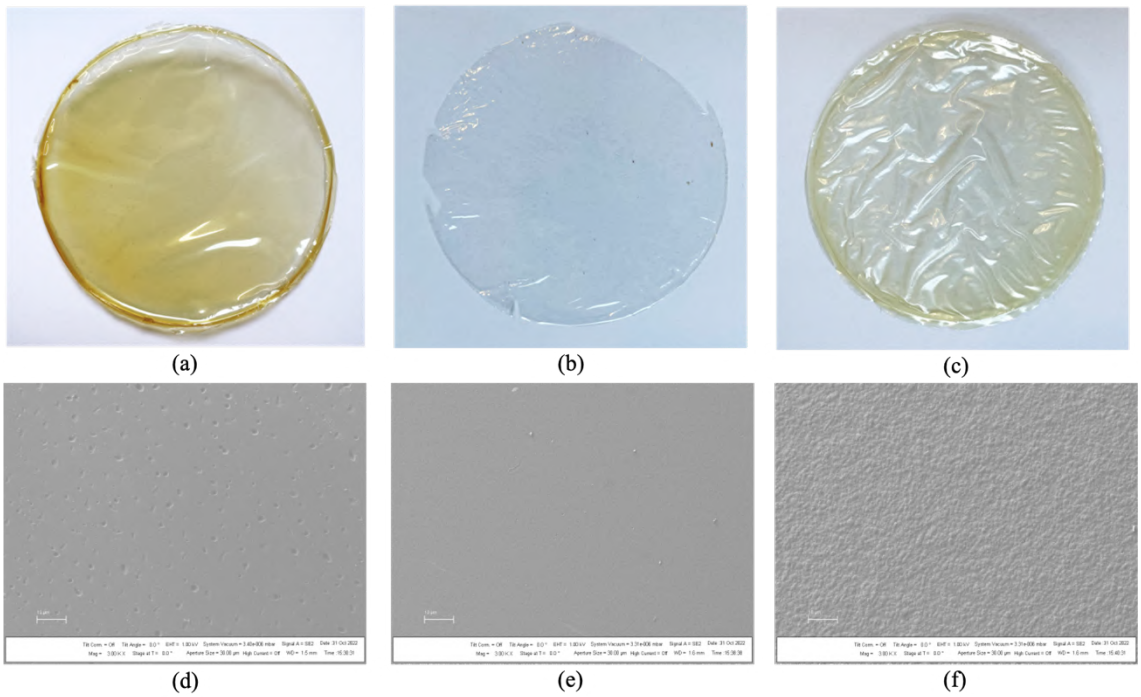


Figure 57: (a-d) zein 20% w/w film sample and FE-SEM image, (b-e) chitosan 2% w/v film sample and FE-SEM image, (c-f) blend zein 2%-chitosan 4% film sample and FE-SEM image.

3.2.3 Mechanical properties

The samples produced were tested in terms of mechanical properties through tensile tests to investigate the effect of the blend between zein and chitosan with respect to the performance of zein alone. The results of the mechanical tests are reported in Table 21, in which the characteristic parameters, i.e., tensile strength (σ_M), elongation at break (ε_B) and Young's modulus (E), are expressed as mean value ± standard deviation.

Table 21: Mechanical properties of zein, chitosan and zein/chitosan blend films in terms of tensile strength (σ_M), elongation at break (ε_B) and Young's modulus (E).

Sample	**Thickness [μm]**	**σ_M [MPa]**	**ε_B [%]**	**E [MPa]**
Zein	60 ± 8.3	24.55 ± 4.49	1.65 ± 0.49	1966.17 ± 193.59
Chitosan	15 ± 4.6	63.51 ± 11.24	7.62 ± 4.76	2251.58 ± 989.85
Blend	30 ± 7.7	46.83 ± 4.76	9.26 ± 1.99	1815.03 ± 225.61

As can be seen from the data obtained, the tensile strength of all the films was found to be highly above the minimum limits envisaged for packaging films (3.5 MPa) [254].
In particular, the zein samples, for which results comparable to those obtained on the same type of material in the previous paragraph were obtained, were found to be fragile due to the low values of elongation at break and rigid due to the high values of Young's modulus. Chitosan samples showed the highest tensile strength values, lower brittleness than zein but higher stiffness. Instead, the polymeric blend led to the obtaining of intermediate values between those obtained from zein alone and from chitosan alone. The most interesting results to discuss concern the elongation values at the break, in fact, through the production of the polymeric blend it was possible to improve this property compared to zein films and also compared to chitosan alone, just as it was possible to improve the values of modulus of elasticity which in fact were lower in the case of the blend. Furthermore, it must be emphasized the high variability of the results obtained from the chitosan samples which in fact have high standard deviations. Figures 58, 59, and 60 show the stress-strain curves obtained from the tensile tests conducted on 6 specimens for the samples of zein, chitosan, and blend respectively.

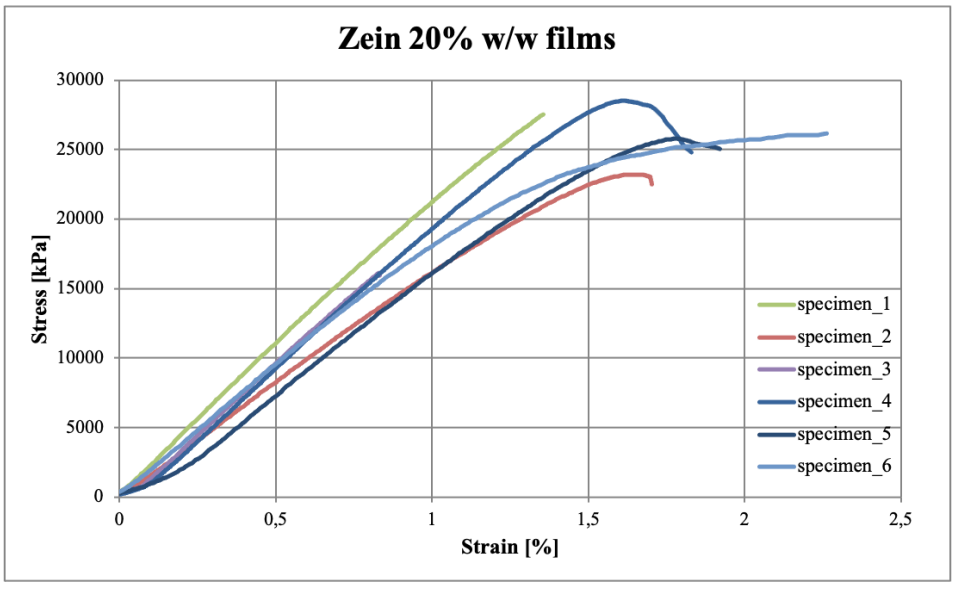


Figure 58: Stress-strain curves of zein 20% w/w films produced by solvent casting.

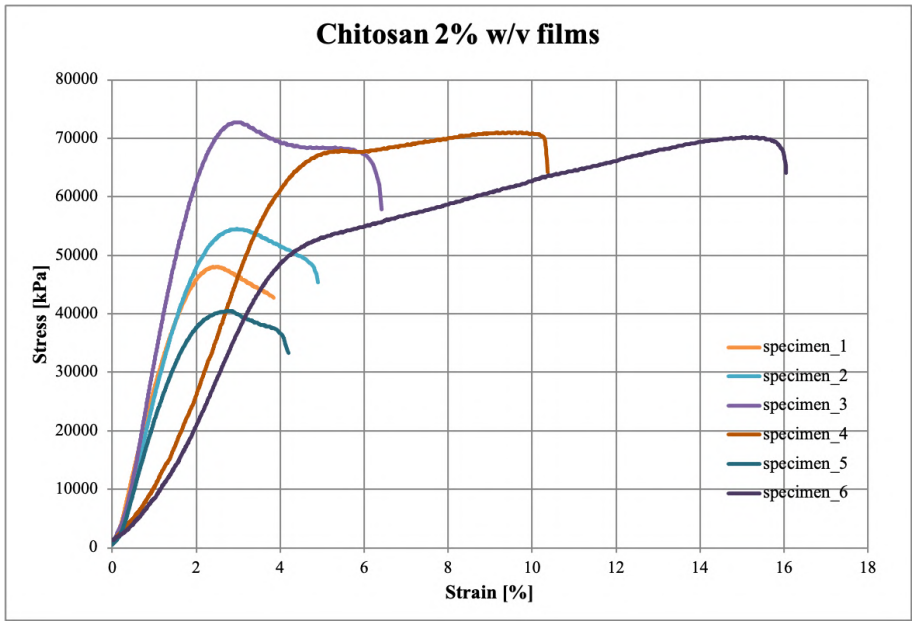


Figure 59: Stress-strain curves of chitosan 2% w/v films produced by solvent casting.

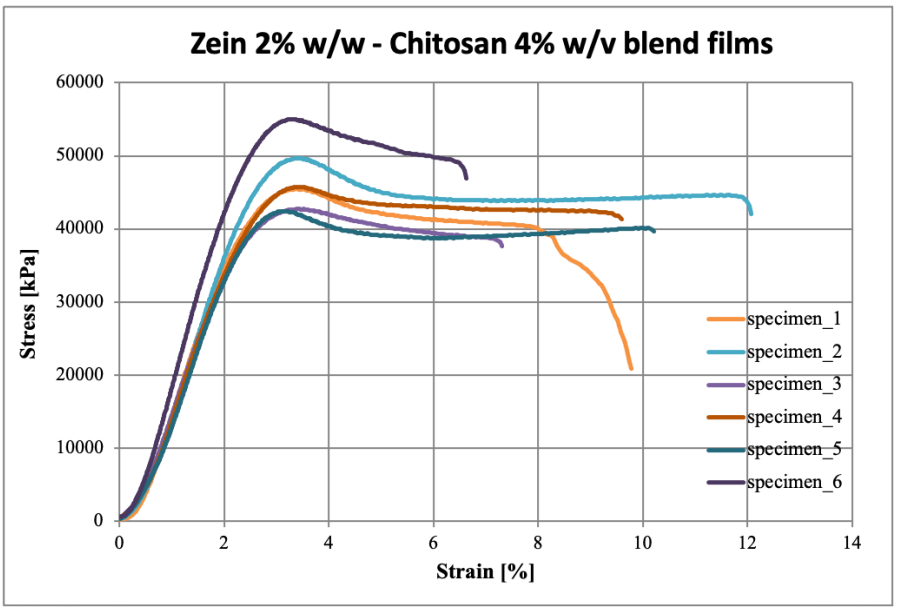


Figure 60: Stress-strain curves of zein/chitosan blend films produced by solvent casting.

As can be seen from Figure 59, the chitosan films are those that have exhibited a more variable behavior, probably due to possible defects in the specimens caused during the preparation phase of the samples due to the very thin thickness. Finally, the blend made it is possible to obtain films with a more marked and more uniform elastic behavior. From the results, it is possible to conclude that

the addition of chitosan to the zein allowed to obtain less rigid, less brittle, and more resistant films with results comparable to those obtained in Yu et al., 2016 [257].

3.2.4 Wettability

The measurement of the contact angle by means of the sessile drop method in static for the evaluation of the surface properties of the samples produced has brought out a very interesting and unexpected fact shown in Figure 61 in which some photos are reported by way of example relating to the measurements of the contact angle for the samples of zein and chitosan evaluated both at the zero instant of deposition of the drop and after 60 seconds that did not show any changes.

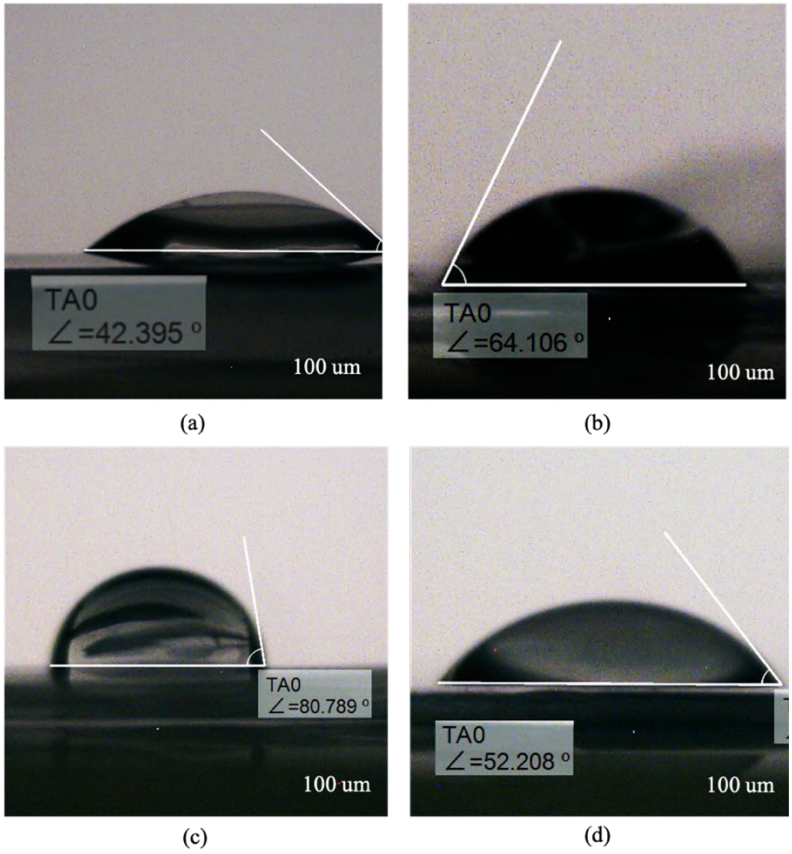


Figure 61: Contact angle (CA) obtained from (a) zein 20% w/w film, (b) chitosan 2% w/v film, (c) blend film at instant zero and (d) blend film after 60 s.

In detail, the films of zein, a protein which by nature is indicated as hydrophobic due to the presence of non-polar amino acids [258,259], were found to have hydrophilic surface characteristics presenting an average value of the contact angle of less than 90° and equal to 42 ± 4.5°, while the

chitosan films, made with this polysaccharide due to its hydrophilic nature, have confirmed to have hydrophilic surface characteristics presenting a contact angle of 67 ± 6.1°. Finally, the blends produced showed a contact angle of 77 ± 4.1° at instant zero, while after 60 seconds the drop deformed reaching a contact angle of 47 ± 10.6°, highlighting a phenomenon of microcapillary of the blend due to the hydrophilicity behavior, also indicating low surface tensions of the liquid. Therefore, the production of the blend did not particularly modify the surface properties obtained from the films of zein alone, in particular, the expected results of decreasing the hydrophobicity of the zein by mixing with a hydrophilic polymer were not obtained. In particular, the hydrophobic/hydrophilic character of the material must not only be associated with the raw polymer but also with the method of production of the samples and the choice of the solvent used, as also discussed by Yu et al., 2016 and Yoshino et al., 2002, in which for example it has been shown that the use of acetone as a solvent for the zein maintains its hydrophobic characteristics if high evaporation rates of the solvent are used, while the use of acetic acid as a solvent for the zein allows improving its mechanical performance in terms of less fragility and rigidity [257,259]. Furthermore, the presence of glycerol in the zein samples, being a hydrophilic plasticizer, contributed to the modification of the surface properties of the samples produced as also reported in [260]. Therefore, to keep the hydrophobic properties of zein unaltered, it would be interesting in future works to investigate the use of other solvents such as acetone for zein and the use of oils as plasticizers.

To conclude, zein has proved to be a promising, versatile material, whose poor mechanical properties can be improved through the production of polymer blends that can be made entirely through the use of biopolymers and non-toxic solvents. The surface properties can be modified, both in the process phase by varying the solvent and the plasticizer and modulating its evaporation rate, or through post-treatments such as corona treatment or cold plasma as illustrated in the introductory chapter. Furthermore, from an economic point of view, as mentioned, the possibility of obtaining resistant films using a negligible quantity of polymer could allow an easier entry of these materials into the market.

4 DEVELOPMENT OF POLYCAPROLACTONE FILMS FOR ACTIVE PACAKGING

PURPOSE

The purpose of this section was to develop biodegradable polymeric films using another polymer, the polycaprolactone, produced by chemical synthesis but still biodegradable. Polycaprolactone, described in paragraph 1.4.5.2, is a polymer of great interest especially in the biomedical field, being accepted from FDA, and it is usually processed by dissolving in solvents such as chloroform. The main challenge of this section was posed by the choice of using only solvents recognized as GRAS, so the first part of the work was dedicated to identifying the best solvent mixture and then moving on to the optimization of the structure and morphology of polymeric films produced mainly by electrospinning. The choice of this technique was made in anticipation of the second part of the work, i.e., the loading of phase change materials (PCMs) inside the polycaprolactone fibers, with the aim of creating a material capable of acting as a thermal buffer to be used in active food packaging in all those products for which compliance with the cold chain is crucial to maintain shelf-life and ensure food safety. As discussed in paragraph 1.5.2, the development of packaging systems capable of counteracting the effect of the inevitable temperature changes along the supply chain is of fundamental importance, but to date, there is still very little literature regarding the encapsulation of PCMs directly inside primary packaging, while containers, refrigerated boxes etc. have been extensively investigated. The novelty of this work is that of having tried to produce an innovative material using polymers, solvents, additives and green processes. The phase change materials selected were two fatty acids, oleic acid and linoleic acid, again to use a green approach to the project, while dodecane was used as a reference material since it is one of the most widely used PCMs. Therefore, the feasibility of their loading in the PCL matrices without structural losses of the polymer films and the product thermal behavior were evaluated. Furthermore, polycaprolactone was processed by impregnation with supercritical fluids (SFI) to investigate the feasibility of loading alpha-tocopherol as an antioxidant agent. The SFI-processed matrices were fabricated by both electrospinning and solvent casting to conduct a comparison between techniques.

4.1. Development and morphological optimization of electrospun PCL processed using green solvents

In this section, the solution and process properties have been investigated in order to produce electrospun films based on polycaprolactone. In particular, the best mixture of solvents for the dissolution of the tested polymer at different molecular weights was first identified and then passed to the study of parameters such as polymer concentration, voltage and process flow rate to conduct a morphological optimization of the fibrous structure of polycaprolactone. This work allowed the identification of the range of process conditions to obtain four types of morphologies: beads, mixed morphology consisting of beads and fibers, micrometric-sized fibers and finally sub-micrometric fibers.

4.1.1 Materials and Methods

4.1.1.1 Solution optimization

The first part of the work was dedicated to identify the most suitable solvent to solubilize ε-polycaprolactone (PCL), purchased by Sigma-Aldrich (Milan, Italy). Both polymer molecular weight and concentration were varied testing 14000-80000 g/mol and 5%, 10%, 20% and 25% w/v with respect to the solvent volume respectively.

Pure acetone, provided by Carlo Erba Reagents (Milan, Italy), and a mixture of acetone and acetic acid, purchased by Sharlau (Barcelona, Spain) were tested in different ratios (1:1, 7:3, 3:7 v/v). For all tests, the solutions were stirred at 60 °C until the complete solubilization of PCL.

4.1.1.2 Optimization of the operative parameters of electrospinning

Once the optimal solution conditions were identified, about 10 mL of each solution were processed by electrospinning. The equipment used to perform the electrospinning process was the Spinbow's Basic Lab Unit (Bologna, Italy), described in the other sections.

Various tests were conducted to investigate the effect of the main electrospinning parameters and their influence on the morphology of the samples produced with the aim of identifying the best set of operative parameters for obtaining a fibrous matrix without defects. In particular, different polymer concentrations were tested (5, 10, 20, 25 % w/v with respect to the solvent volume), varying both the flow rate between 0.5, 0.7, 1.2, 1.7 and 2.2 mL/h, and the voltage between 15, 17 and 19 kV, while the distance between the needle and the collector was kept fixed at 17.5 cm using a 18-gauge needle. Once the process was complete, the samples were manually removed from the collector and stored at room temperature.

4.1.1.3 Morphology characterization

The samples were analyzed by Scanning Electron Microscopy (SEM, Hitachi S-2500), without any pretreatment, using an accelerating voltage of 10 kV. Multiple images at different enlargements were collected for each sample.

The images obtained by SEM were then analyzed using ImageJ and Origin 9.1 (Microcal, Northampton, MA, US) software to estimate the fibers size distribution. For each image, the diameters of at least 300 fibers were measured. A digital thickness gauge (PosiTector 6000) was used to measure the thickness of the samples. For greater accuracy, at least five measurements were taken for each sample and the results were expressed as mean value ± standard deviation.

4.1.2 Results and discussion

4.1.2.1 Solution optimization tests

The main purpose of this step was to identify an alternative solvent to those commonly used to dissolve PCL, which was less toxic and more environmentally friendly.

Initially, only acetone was tested, but the PCL, for neither of the two molecular weights tested, achieved complete solubilization. Then, different mixtures of acetone and acetic acid (1:1, 7:3, 3:7 v/v) were tested as reported in [261,262]. Complete dissolution for both molecular weights (14000-80000 g/mol) and for all the concentrations investigated (5, 10, 20 and 25% w/v) was obtained for the mixture of acetone and acetic acid in a ratio equal to 3:7 v/v, which consequently were selected for electrospinning process.

To study the influence of process parameters on the morphology of polymeric films, all tests conducted by electrospinning were systematically analyzed by SEM. The set of tests carried out in this part of the work is summarized in Table 22, which shows the preliminary results obtained regarding the solution and the morphology appearance.

The first important consideration is related to the molecular weight of the polymer, in fact, for the tests with PCL at lower molecular weight, equal to 14.000 g/mol, the conditions for conducting an electrospinning process were not obtained, but rather an electrospray process was performed. In fact, particles were produced instead of fibers. The same result was obtained for PCL solutions with a molecular weight of 80.000 g/mol, at the lowest polymer concentration tested, 5% w/v.

Identifying the correct molecular weight and the right polymer concentration is of fundamental importance as these two parameters influence the formation of the Taylor cone. In fact, for low molecular weight and concentration values, there is no formation of the Taylor cone and the electrohydrodynamic process acts as an electrospray with the atomization of the solution [263]. While for the electrospinning process the formation of the Taylor cone is required, and this can only

occur at concentrations and molecular weights above a certain threshold which obviously changes as the polymer used varies.

Table 22: Experimental set of electrospinning parameters and outputs in terms of solution and morphology appearance.

PCL Mw [g/mol]	PCL Concentration [% w/v]	Solvent acetone/acetic acid ratio	Solution appearance	Voltage [kV]	Flow rate [ml/h]	Morphology appearance
14000	5-10-20-25	1:0	Not solubilized	-	-	-
14000	5-10-20-25	1:1	Not solubilized	-	-	-
14000	5-10-20-25	7:3	Not solubilized	-	-	-
80000	5-10-20-25	1:0	Not solubilized	-	-	-
80000	5-10-20-25	1:1	Not solubilized	-	-	-
80000	5-10-20-25	7:3	Not solubilized	-	-	-
14000	5-10-20-25	3:7	Solubilized	15	1.2-2.2	Particles
80000	5	3:7	Solubilized	15	1.2-2.2	Particles
80000	10	3:7	Solubilized	15-17	0.5-0.7	Beads
80000	10	3:7	Solubilized	17	1.7-2.2	Mixed
80000	20	3:7	Solubilized	17	1.7	Fibers
80000	20	3:7	Solubilized	19	1.7	Fibers
80000	25	3:7	Too viscous	-	-	-

The tests carried out with PCL (80000 g/mol) at a concentration equal to 10% led to the formation of beads for low voltages and flow rates, while increasing these parameters a mixed morphology was obtained. The SEM images relating to these two morphologies are shown in Figure 62.

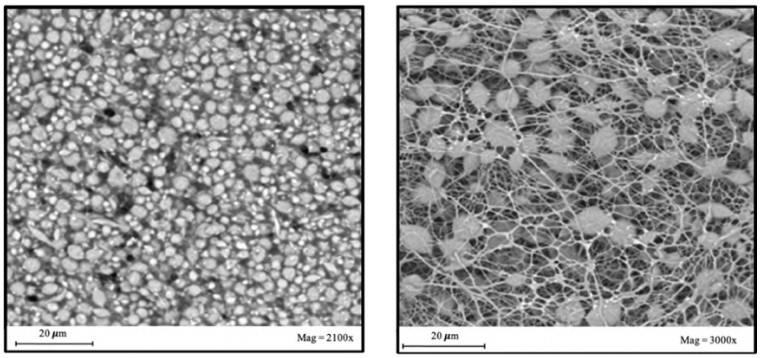


Figure 62:SEM images of PCL (a) beads and (b) mixed morphology.

Only by working at a concentration equal to 20% of PCL it was possible to obtain fibers whose size depended on the voltage and the flow rate. In fact, for low flow rate and voltages micrometric fibers with a mean diameter equal to 1.26 ± 0.72 μm were obtained, while for higher voltages and flow rate, sub-micrometric fibers were obtained with a mean diameter equal to 0.85 ± 0.54 μm as shown in Figure 63.

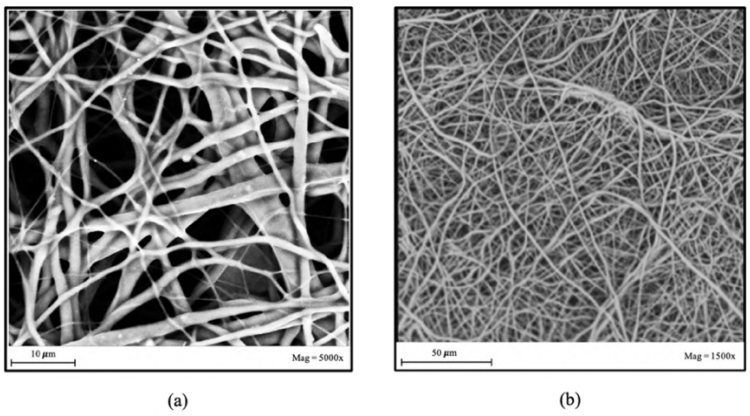


Figure 63:SEM images of (a) micrometric and (b) sub-micrometric electrospun PCL.

Furthermore, for concentrations greater than 20% w/v, in particular when testing 25% PCL solutions, it was not possible to carry out the electrospinning process as the solution was too viscous. The summary of this morphological optimization study is shown in Figure 64.

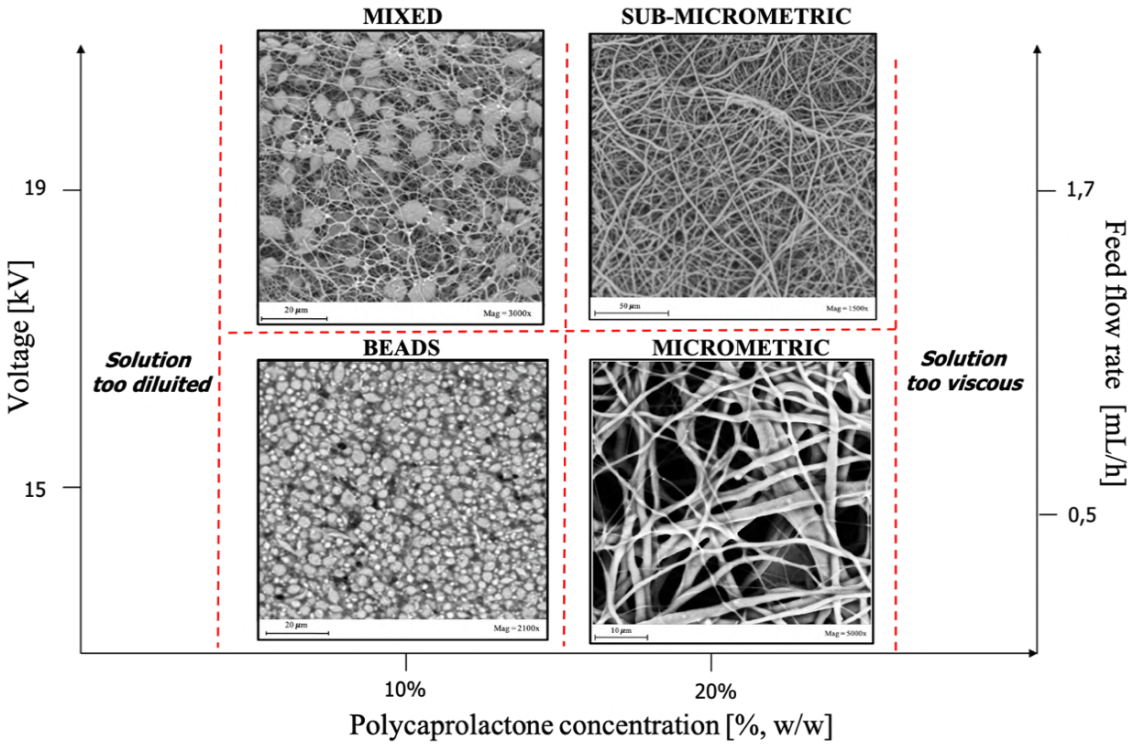


Figure 64:Morphological optimization of the electrospun PCL samples.

In particular, this study allowed the identification of four morphologies of the polymeric structures at the variation of the three most important parameters of the electrospinning, and the identification of the operational limits of the process. In summary, the set of optimal parameters identified was the following: PCL (80000 g/mol) with a concentration of 20% w/v with respect to the volume of solvent consisting of a mixture of acetone and acetic acid in a 3:7 v/v ratio, processed with a feed flow rate of 1.7 mL/h, an applied voltage of 19 kV and a needle-collector distance equal to 17.5 cm. Finally, Figure 65 shows a macroscopic image of an electrospun PCL film. In particular, it was possible to obtain films with a mean thickness of 92.20 ± 11.28 µm.

Figure 65:Image of optimized electrospun PCL sample.

4.2 Loading of fatty acids as potential phase change materials in electrospun PCL

In this section, the solution and process parameters identified as the optimal were used to develop an electrospun PCL films loaded with two fatty acids, linoleic acid and oleic acid, and a paraffin, dodecane used as control. The main goals were to verify if the loading of these compounds influences the previously optimized morphology of the fibers or not, and to obtain films with a potential use in the field of active packaging with thermal insulation properties. In particular, linoleic acid and dodecane, have a melting temperature of -10 °C, and therefore they could be useful for frozen food thermal protection. Instead, oleic acid having a melting temperature of about 4-8 °C, could be used for refrigerated food. Through this study it was possible to successfully obtain the loading of these compounds without altering the morphology of the polycaprolactone fibers. Furthermore, in the literature, for the best of our knowledge, there are no other works that have studied the feasibility of loading these fatty acids in polycaprolactone fibers and its effect. The thermal behavior results underlined two aspects. First of all, it was possible to notice the establishment of bonds between polyester and fatty acids, confirming the possibility of obtaining a non-migratory active material. Furthermore, it was found that the quantities of PCMs loaded were too small to be able to perform even the expected action of phase change materials, as the bonds with the polymer did not leave the possibility for the PCM to be free to carry out their function. Therefore, the possible future solutions could be two, either to increase the load of PCMs or, even better, to encapsulate the fatty acids in inert nanoparticles before producing the enriched PCL films, so as to leave them free to change phase but at the same time avoid their own migration outside the fibers.

4.2.1 Materials and Methods

4.2.1.1 Loading of phase change materials

A feasibility study of the PCMs loading on the optimized PCL support was performed to verify the influence of these compounds on the morphology and features of the polycaprolactone fibers.

For the production of the loaded samples, linoleic acid, oleic acid and dodecane, purchased by Sigma-Aldrich (Milan, Italy), were individually added to the solutions in different percentages equal to 10%, 20%, and 30% by weight with respect to the polycaprolactone content (20% w/v with respect to the solvent volume). Specifically, the solutions were heated to 60 °C and held at this temperature for at least 30 minutes and then cooled to room temperature before processing. Approximately 6 mL of each solution was electrospun using the best parameter set identified in the previous section with soft modification, i.e., the flow rate was set at 1.2 mL/h, while the voltage was maintained at 19 kV, the 18-gauge needle-collector distance at 17.5 cm. Each test was performed in triplicate and the samples

were collected and stored at room temperature before being analyzed. The experiments conducted are listed in the following Table 23.

Table 23:PCMs loading into PCL fibers experimental set.

Sample	PCL [% w/v]	Solvent [mL]	PCM	PCM [% w/w]
Blank	20	10	-	-
D10	20	10	Dodecane	10
D20	20	10	Dodecane	20
D30	20	10	Dodecane	30
LA10	20	10	Linoleic acid	10
LA20	20	10	Linoleic acid	20
LA30	20	10	Linoleic acid	30
OA10	20	10	Oleic acid	10
OA20	20	10	Oleic acid	20
OA30	20	10	Oleic acid	30

4.2.1.2 Morphology characterization

All the samples were analyzed by Hitachi S-2500 Scanning Electron Microscopy apparatus (SEM), without any pretreatment. The images obtained by SEM were then analyzed using ImageJ and Origin 9.1 (Microcal, Northampton, MA, US) software to estimate the fibers size distribution. For each image, the diameters of at least 300 fibers were measured. A digital thickness gauge (PosiTector 6000) was used to measure the thickness of the samples. For greater accuracy, at least five measurements were taken for each sample and the results were expressed as mean value ± standard deviation.

4.2.1.3 Mechanical properties

Mechanical tests were conducted on all the samples produced using the Zwick Roell Z0.5 equipment in combination with Zwick Roell's testXpert III software. To perform the tensile tests, five dog-bone specimens of size 5x20 mm^2 were made from each sample.

A tensile speed of 10 mm/min, an initial grip separation of 21 mm and 500 N load cells were used for the analysis.

4.2.1.4 Thermal analysis

The thermal behavior of the sample produced at the highest PCMs concentration (30% w/w) was evaluated using a differential scanning calorimeter (Mettler, DSC-821e). Specifically, 8 mg of each sample were subjected to three consecutive thermal cycles of heating from -20 °C to 70 °C with a heating rate of 2 °C/min in a nitrogen atmosphere (40 mL/min), and then cooled at a rate of -10 °C/min.

4.2.1.5 Migration tests

In order to verify to have obtained non-migratory systems, migration tests were conducted using an hydroalcoholic solution of ethanol 10% v/v as simulant for food with hydrophilic character. The tests were conducted on the samples loaded with linoleic acid, as the developed system proved to be more reproducible in terms of morphology and mechanical properties than the other PCMs. A fixed surface of the sample, equal to 6.25 cm^2, was immersed in the simulant and left to soak at room temperature for 14 days with exposure to artificial light to simulate the worst storage conditions for refrigerated food. At fixed time intervals, the concentration of linoleic acid within the simulant fluid was analyzed via UV-vis analysis, using the Perkin Elmer Spectrophotometer (model Lambda 25), after calibration, at 280 nm [264], and expressed as mg of linoleic acid released for mg of linoleic acid theoretically loaded into the films by electrospinning.

4.2.2 Results and Discussion

4.2.2.1 Feasibility study of fatty acids loading by electrospinning

The selected PCMs were linoleic acid, oleic acid and dodecane, which were loaded by varying their concentration in the polymer solution between 10%, 20% and 30% by weight with respect to the polymer content, set at 20% w/v. The loaded samples were produced with a slightly lowered flow rate, 1.2 mL/h, respect to the unloaded PCL, due to the formation of a more unstable jet after the Taylor cone caused by the slight changes in the solution induced by fatty acids, probably in terms of viscosity and electric conductivity. All the tests led to the formation of more than satisfactory films and the effect of the presence of fatty acids was already visible from the macroscopic structure, as shown in Figure 66. In fact, unlike the samples of only PCL (blank sample) and of PCL loaded with dodecane, the loading of fatty acids has led to the formation of films with a much rougher surface, attributable to a higher electrostatic charge on the collected fibers.

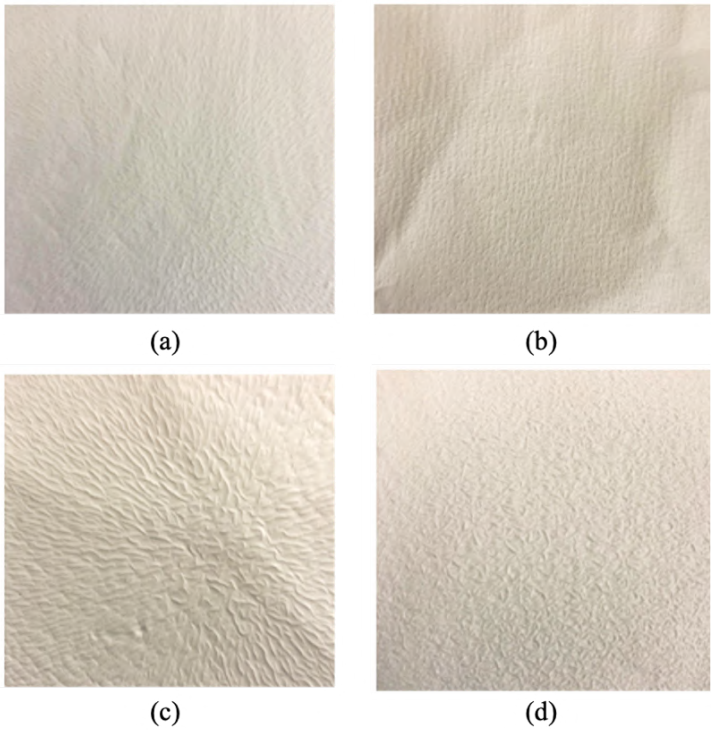


Figure 66: Differences in sample surfaces depending on the type of PCM used: (a) blank sample, (b) dodecane 30% w/w, (c) linoleic acid 30% w/w and (d) oleic acid 30% w/w.

In Table 24 the main characteristics of the films in terms of thickness and mean fibers diameter (MD) are reported and expressed as mean value ± standard deviation. All the samples had a rather irregular thickness between 85.00 ± 8.17 μm and 335.80 ± 46.21 μm, as well as a wide distribution of fiber diameters both in sub-micrometric and micrometric scale.

Table 24: Results of film thickness and mean fibers diameter (MD) evaluation.

Sample	Thickness [μm]	MD [μm]
Blank	92.20 ± 11.28	0.85 ± 0.54
D10	108.75 ± 29.84	0.97 ± 0.47
D20	98.00 ± 16.19	0.64 ± 0.36
D30	85.00 ± 8.17	0.73 ± 0.43
LA10	164.40 ± 38.79	0.91 ± 0.53
LA20	197.80 ± 41.38	0.87 ± 0.43
LA30	274.60 ± 34.89	1.76 ± 0.73
OA10	143.00 ± 60.97	1.01 ± 0.48
OA20	335.80 ± 46.21	0.90 ± 0.40
OA30	259.40 ± 64.99	1.79 ± 0.82

A considerable increase in the thickness of the films was noted, with the same volume processed, in the case of the samples loaded with fatty acids due to a smaller deposit surface of the fibers, not due to the collector, but to the behavior of the material itself, always attributable to the different electric behavior respect to the blank sample.

Instead, no effect of the loaded compounds on the mean diameter of the fibers was noted, other than an increase in the mean diameter compared to the blank sample for the samples loaded with fatty acids at the higher concentration. Looking at the SEM images in Figure 67, no significant differences were observed respect to the morphology of the blank sample (Figure 63b). The addition of the PCMs, therefore, did not result in a significant change in sample structure, even at high percentages of PCM 30% w/w.

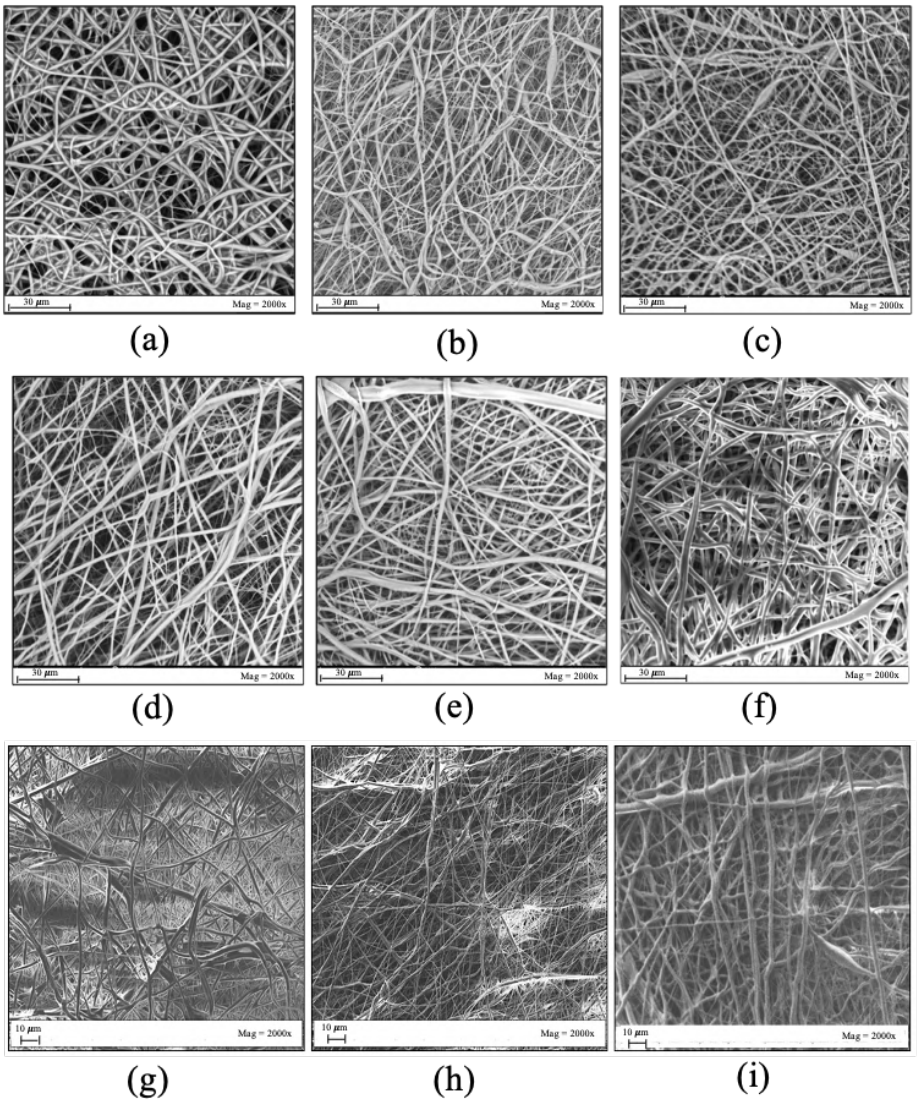


Figure 67: SEM images of electrospun PCMs loaded samples in PCL support: D10 (a), D20 (b), D30 (c), LA10 (d), LA20 (e), LA30 (f), OA10 (g), OA20 (h), OA30 (i).

4.2.2.2 Mechanical properties

Since the samples produced by electrospinning are porous materials, it was not considered useful to evaluate the gas permeability properties, having already observed through the analyzes carried out on the electrospun zein that these polymeric structures are completely permeable to gases. Instead, it was considered interesting to investigate the mechanical properties of the electrospun polycaprolactone films which, unlike the zein samples, were found to be suitable for analysis through tensile tests. All the samples produced were analyzed to assess whether there was an influence of the loaded compounds on the mechanical properties in terms of tensile strength (σ_M), elongation at break (ε_B) and Young's modulus (E). Since the samples are isotropic, as evidenced by the SEM images that showed the random orientation of the fibers, the tensile tests were conducted in a single direction and the behavior of the samples during the test is reported in Figure 68.

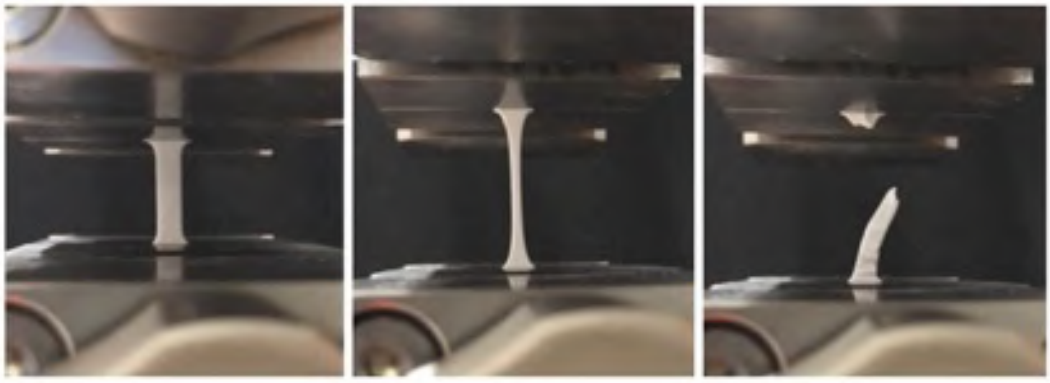


Figure 68: Picture of the specimen before (left), during (center) and after (right) the tensile test.

The results of the tensile tests relating to all the samples are summarized in Table 25.

As general result, according to conventional standards, the tensile strength value of a material should be at least 3.5 MPa in order to be use as food packaging. In these terms, all the samples produced meet the requirement except those with the highest thicknesses: LA30, OA20 and OA30 for which the tensile strength was below this limit.

Table 25: Mechanical properties of sample in terms of tensile strength (σ_M), elongation at break (ε_B) and Young's modulus (E), expressed as mean value ± standard deviation.

Sample	σ_M [MPa]	ε_B [%]	E [MPa]
B	4.92 ± 1.08	225.26 ± 42.77	31.97 ± 10.41
D10	6.63 ± 1.74	165.80 ± 7.23	14.57 ± 6.72
D20	10.56 ± 1.19	132.76 ± 6.15	18.27 ± 6.19
D30	9.44 ± 2.84	111.47 ± 6.79	15.78 ± 5.27
LA10	4.76 ± 1.52	75.40 ± 6.28	9.83 ± 2.70
LA20	4.15 ± 0.70	85.81 ± 3.93	9.61 ± 1.70
LA30	2.91 ± 0.18	75.16 ± 9.16	6.94 ± 1.66
OA10	5.27 ± 2.01	298.50 ± 63.28	19.37 ± 9.33
OA20	2.66 ± 0.22	107.01 ± 14.56	6.46 ± 0.70
OA30	2.56 ± 0.64	77.49 ± 10.40	6.86 ± 2.62

The most evident effect of PCMs loading was on the value of Young's modulus, which proved to be much lower than that of the blank sample for all the PCMs loading, demonstrating that the fatty acids and dodecane make the material less rigid. As regards the elongation at break, all the loaded samples presented a value of this parameter lower than that of the blank sample, except in the case of OA10 which proved to be the least fragile. Figures 69 show the stress-strain curves obtained as the phase change material and the concentration loaded in the polymer varies.

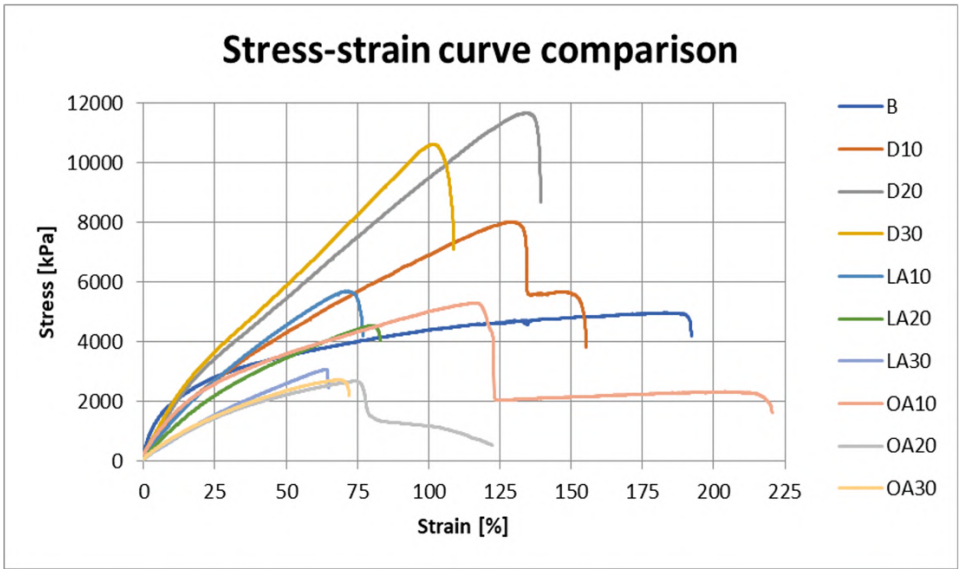


Figure 69: Stress-strain curves of the specimens compared.

It is interesting to note the behavior of some samples, such as OA10, OA20 and D10, for which the curves show a step relative to the breaking of the samples by layers. In fact, the electrospun samples are made up of many layers of overlapped fibers which, in some cases, have broken and flaked apart but this behavior is independent from the PCMs loading. Furthermore, of all the samples those that showed more repeatability were the linoleic acid samples in all percentages. To show the wide variability of the specimens belonging to the same class, in the Figures from 70 to 72, the stress-strain curves of the single specimen are reported in comparison with the blank sample.

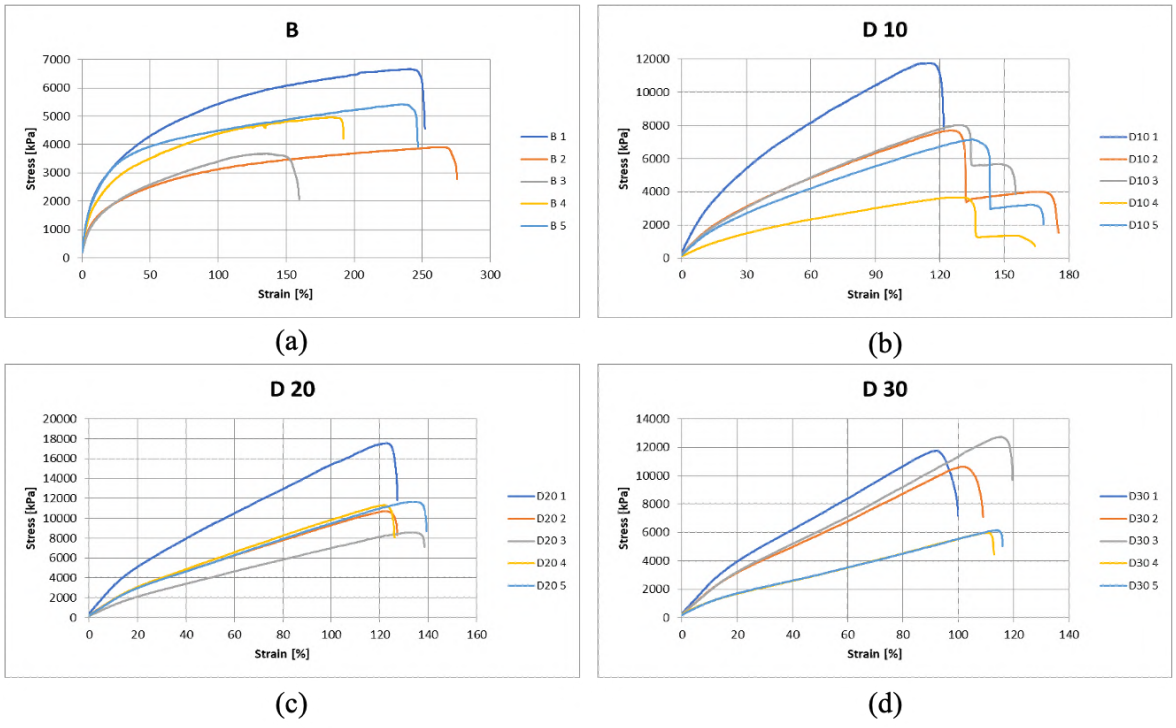


Figure 70:Stress-strain curves of (a) blank sample, (b) PCL loaded with dodecane 10% w/w, (c) PCL loaded with dodecane 20% w/w and (d) PCL loaded with dodecane 30% w/w.

Compared to the samples with only PCL (blank), the samples loaded with dodecane showed higher tensile strength, lower stiffness but greater brittleness.

While, films produced with the loading of linoleic acid exhibited similar or slightly lower tensile strength, much lower stiffness and greater brittleness.

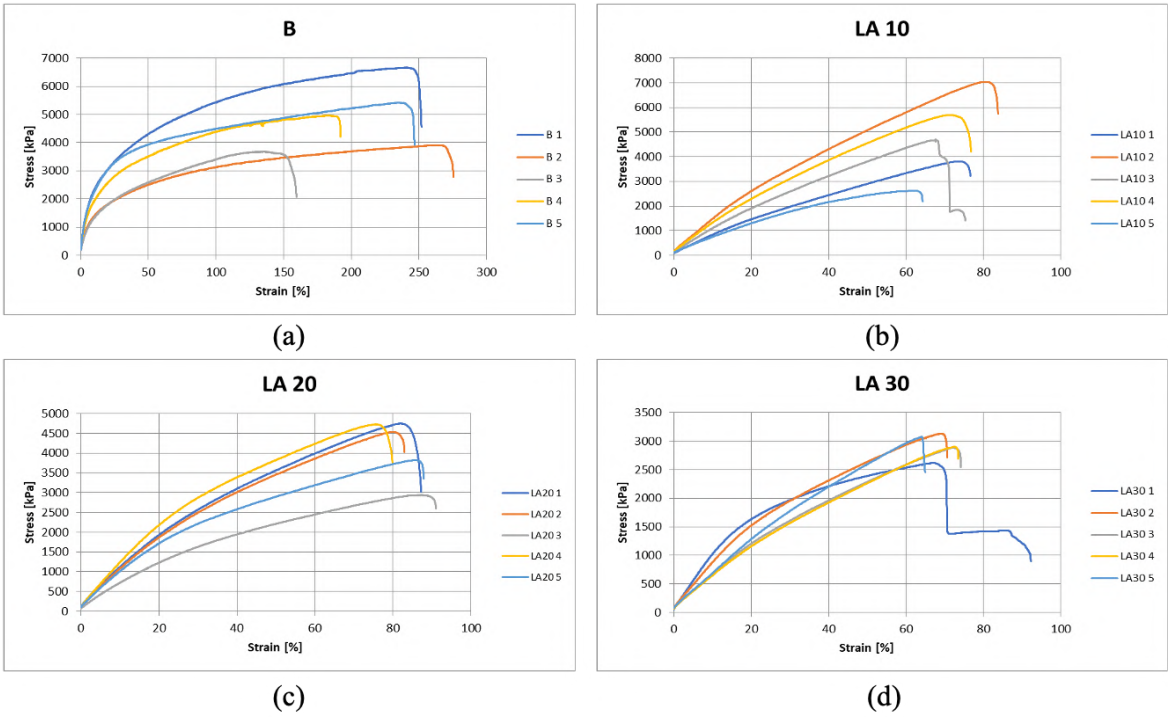


Figure 71: Stress-strain curves of (a) blank sample, (b) PCL loaded with linoleic acid 10% w/w, (c) PCL loaded with linoleic acid 20% w/w and (d) PCL loaded with linoleic acid 30% w/w.

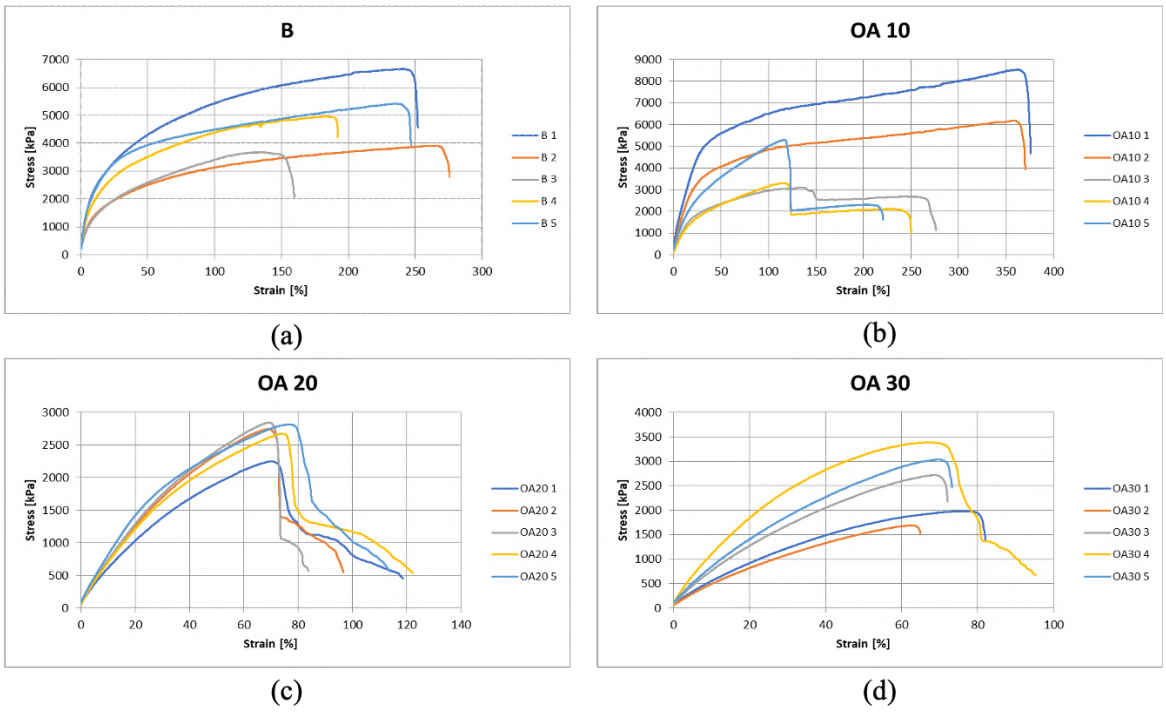


Figure 72: Stress-strain curves of (a) blank sample, (b) PCL loaded with oleic acid 10% w/w, (c) PCL loaded with oleic acid 20% w/w and (d) PCL loaded with oleic acid 30% w/w.

Finally, the samples loaded with oleic acid, compared to the blank sample, showed lower tensile strength, especially for higher loads, similar brittleness and lower stiffness. With regard to the results obtained on the blank PCL sample, although it is difficult to make a direct comparison with the results obtained by other authors, as these depend on the solvent used, the process conditions, the type of collector and the thickness of the films produced beyond that from the size of the fibers, it can however be noted that the PCL films produced in this work have higher values of tensile strength, elongation at break and Young's modulus than those obtained by [157,262], in whose work was used PCL at the same molecular weight, with similar solution and process conditions. It is also interesting to compare it with another well-known and commonly used biopolymer, polylactic acid (PLA). Leones et al. produced fibrous samples of PLA by electrospinning [265]. PLA was found to be stiffer than PCL by having a significantly higher Young's modulus, while tensile strength and elongation at break were found to be higher for PCL than for PLA.

4.2.2.3 Thermal analysis

The DSC thermograms obtained are represented in Figure 73 and show the curves relating to the heating and cooling cycles of the blank sample and of the samples loaded with the highest percentage of PCMs, i.e., 30% w/w with respect to the polymer content.

In particular, the heating curves of the PCL sample show an endothermic peak at about 57-61 °C relative to its melting temperature, while, in cooling phase, an exothermic peak at about 34 °C represents the crystallization temperature of polycaprolactone.

More interesting, looking at the DSC thermograms of samples loaded with linoleic and oleic acid, it can be noted that the amplitude of the curves is larger than the PCL, demonstrating an increase in the temperature range in which the PCL phase change occurs. In detail, in the case of linoleic acid, the melting temperature is slightly lowered between 55 and 57 °C, and the effect in the cooling phase is greater with a peak at 25 °C relating to the crystallization phase. While in the case of oleic acid the crystallization peak is below 30 °C. This effect is probably due to the establishment of chemical bonds between the polyester and the unsaturated fatty acids that, by binding to the polymer, are not free to change phase independently of the polymer, in fact, other characteristic peaks of pure fatty acids were not noticed.

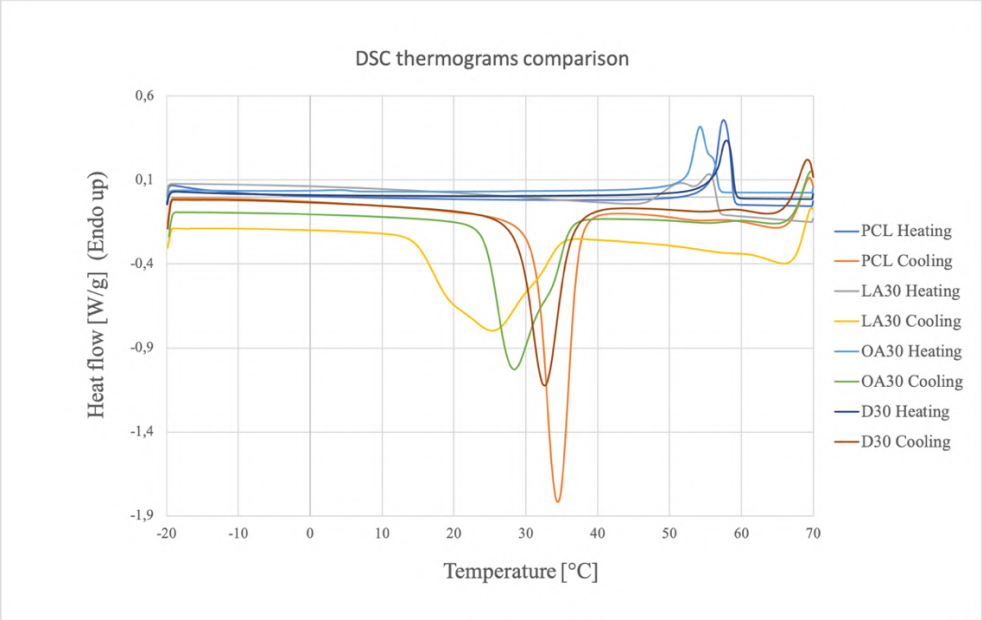


Figure 73: DSC thermograms relative to the second thermal cycle of PCL and PCL loaded at the highest percentage of PCMs.

In Table 26 are reported the thermal characteristics obtained from each thermal cycle in terms of melting and crystallization temperature (T_m and T_c), fusion enthalpy during the heating scan (ΔH_f) and crystallization enthalpy during the cooling scan (ΔH_c).

Table 26: DSC heating and cooling characteristics of PCL and loaded PCL.

Sample	**Thermal Cycle**	**T_m [°C]**	**ΔH_f [J/g]**	**T_c [°C]**	**ΔH_c [J/g]**
PCL	1°	61	60.78	34	-46.82
PCL	2°	57	44.29	34	-47.31
PCL	3°	57	46.14	34	-47.06
D30	1°	60	47.17	32	-35.95
D30	2°	58	36.44	33	-36.45
D30	3°	58	37.42	33	-36.37
AL30	1°	57	59.06	25	-43.28
AL30	2°	55	45.81	25	-43.30
AL30	3°	55	44.92	25	-43.66
AO30	1°	58	56.23	28	-39.71
AO30	2°	54	40.84	28	-40.04
AO30	3°	54	40.76	28	-39.88

The curves, for each sample, were found to be superimposable during the three thermal cycles as reported in Figures 74-76, in which the three thermal cycles of unloaded PCL and PCL loaded with the three different phase change materials are reported for comparison.

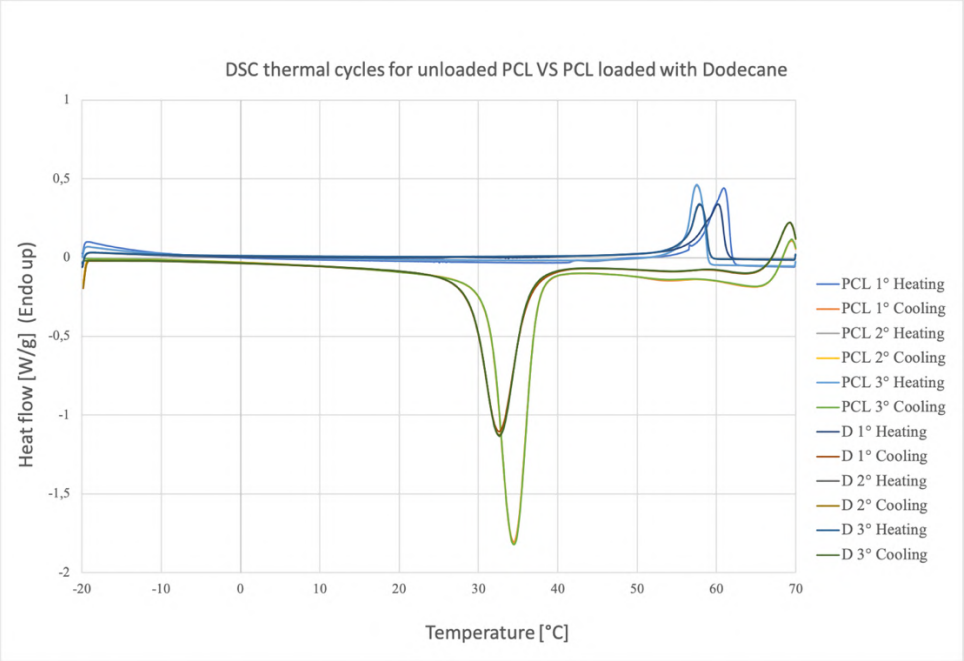


Figure 74: DSC thermograms of three thermal cycles for PCL and PCL loaded with dodecane 30% w/w.

In particular, as can be seen from Figure 74, in the case of the polycaprolactone samples loaded with dodecane 30% w/w, no effect was observed on the amplitude of the peak related to the change in state of the polymer, neither in the melting phase nor in the crystallization phase. The relative peaks were simply shifted.

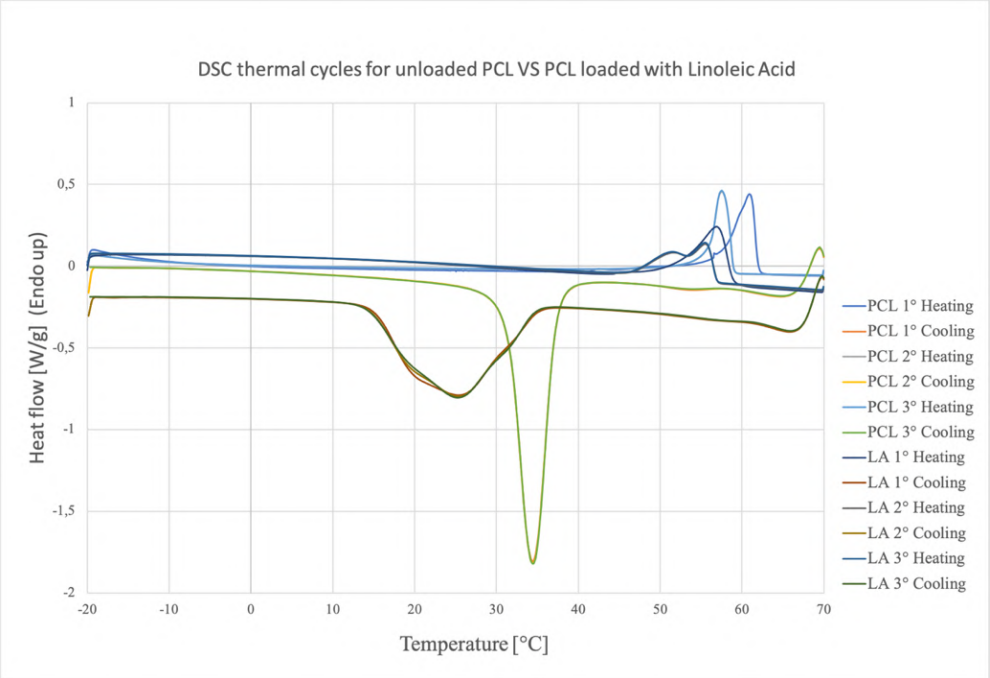


Figure 75: DSC thermograms of three thermal cycles for PCL and PCL loaded with linoleic acid 30% w/w.

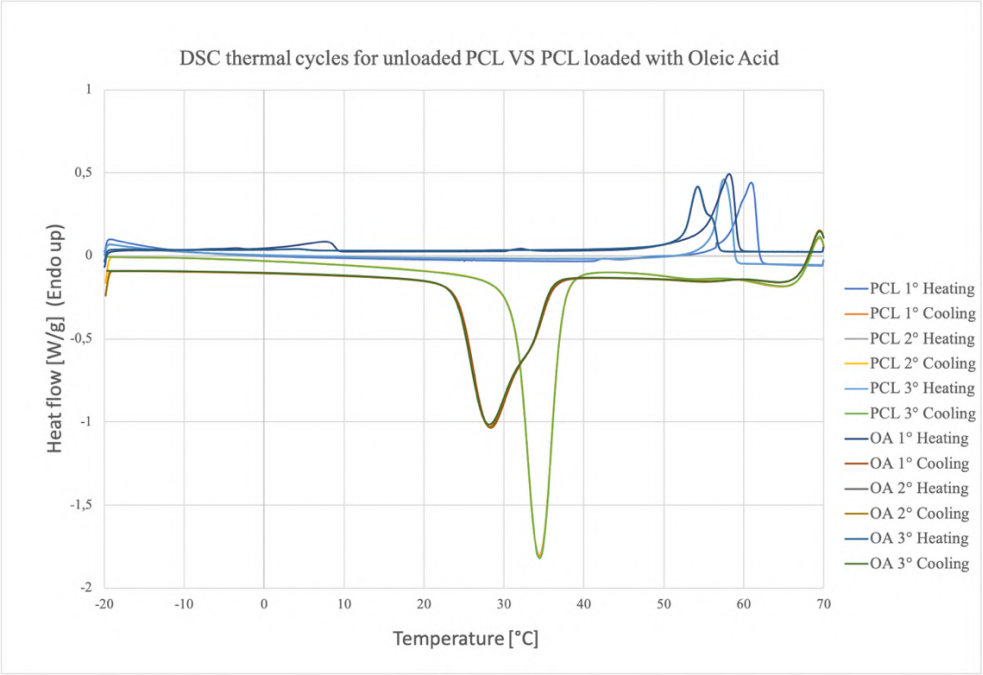


Figure 76: DSC thermograms of three thermal cycles for PCL and PCL loaded with oleic acid 30% w/w.

Instead, from Figure 75 and Figure 76 the increase in the crystallization temperature range relative to polycaprolactone, but not to individual fatty acids, can be noted. Only in the case of oleic acid

there was a small peak observed around 8 °C which could be associated with the melting temperature of oleic acid.

The increase in the range of temperatures at which the polymer changes phase could be an interesting result for others applications.

These results underline the need to increase the concentration of the loaded PCMs, in order to saturate the bonds. Or, an interesting solution could be to encapsulate the PCM in nanoparticles of another polymer inert towards them and then produce the fibers by electrospinning so as not to let the PCMs come into contact with the polycaprolactone in order to prevent the establishment of bonds with this polyester.

4.2.2.4 Migration test

The migration tests of linoleic acid from the electrospun PCL were conducted by total immersion method in static way, using an hydroalcoholic solution of ethanol 10% v/v for 14 days at room temperature. The release fluids were analyzed via UV-vis analysis at fixed time intervals, after 24 h, 48 h and 14 days. The results expressed as mg of linoleic acid released with respect to the mg of linoleic acid theoretically loaded are reported in Figure 77 for all the three percentages of loaded linoleic acid, i.e., 10%, 20% and 30% w/w. As can be seen, the release of linoleic acid is very low if compared to the other compounds loaded into polymeric matrices through electrospinning technique discussed in this work. In fact, the percentage of linoleic acid released was less than 2% for all the linoleic acid load. This result was a further confirmation of what was revealed by the thermal analyzes, i.e., that the fatty acids used, being unsaturated, created chemical bonds with the polyester, allowing to obtain non-migratory films, a fact not taken for granted considering that up to now the electrospinning technique was used, in this work, precisely to obtain high release kinetics. It is however necessary to underline that this study is still in a preliminary phase and that further investigations will be necessary to confirm the non-migratory character, in particular, it will be necessary to test other more aggressive food simulants towards fatty acids, such as hydroalcoholic solutions of ethanol with percentage higher than 10% and conduct more specific analyzes, for example by gas chromatography to confirm this behavior also for the other samples.

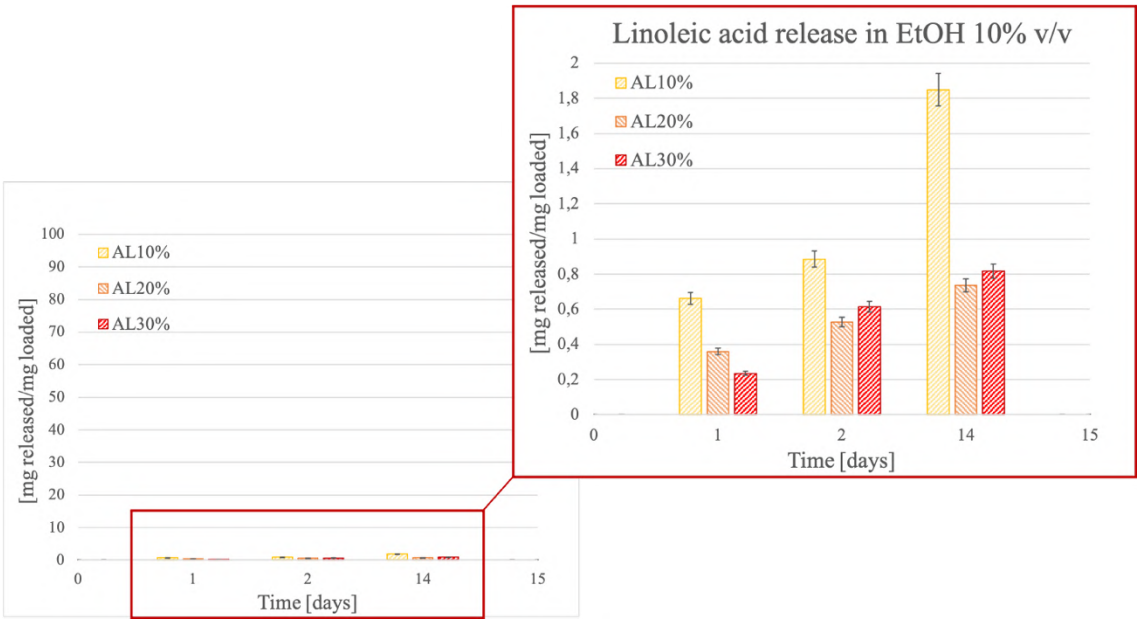


Figure 77:Linoleic acid released from PCL fibers after 1, 2 and 14 days of contact with ethanol 10% v/v at room temperature.

This work, albeit in a preliminary phase, has made it possible to obtain the encapsulation of various phase change materials, including dodecane, chosen as paraffins, as they are among the most used PCMs even if not pure but in blends, while linoleic and oleic acids have been selected as natural fatty acids with phase change temperatures suitable for food application. The PCMs encapsulation was successful without altering the morphology of the polycaprolactone fibers, requiring a small change in the feed flow rate during the electrospinning process. Furthermore, this study is quite innovative both in terms of the final application envisaged and in the choice of materials, both as regards the polymer and additives, both for the solvents used and for the process adopted, despite the need for improvements already identified.

4.3 Loading of alpha-tocopherol in different optimized PCL supports by supercritical fluids impregnation

The data that will be discussed in this section has been already published:

- E. Drago, R. Campardelli, I. De Marco, P. Perego. Optimization of PCL Polymeric Films as Potential Matrices for the Loading of Alpha-Tocopherol by a Combination of Innovative Green Processes. *Processes*, 2021, 9, 2244. Doi: 10.3390/pr9122244.

The aim of this section was to perform and compare the supercritical fluids impregnation (SFI) of an antioxidant compound, alpha-tocopherol, on two different polycaprolactone matrices, nanofibrous structures obtained by electrospinning and continuous structures obtained by solvent casting. The best solution and process conditions for subjecting the samples to the SFI process were identified. In particular, the different morphologies of the samples both before and after the supercritical impregnation process were investigated, identifying the limits and possible solutions to obtain an optimization of the constructs to be impregnated with this innovative green technology in the packaging field. The comparison between the different materials developed was performed in terms of alpha-tocopherol release kinetics.

4.3.1 Materials and Methods

4.3.1.1 Materials

Polycaprolactone (PCL, Mn 80000), alpha-tocopherol (purity > 97%), and polyethylene glycol (PEG 400) were purchased by Sigma-Aldrich (Milan, Italy); acetone and acetic acid glacial were provided by Carlo Erba Reagents S.A.S (France); carbon dioxide (CO_2, purity 99%) was purchased by Morlando Group s.r.l. (Sant'Antimo, Naples, Italy).

4.3.1.2 Production of PCL supports by solvent casting

As regards the solvent casting process, the solutions were prepared at a concentration of polycaprolactone equal to 20% w/v dissolved in a solution of acetone and acetic acid in a ratio of 3: 7 v/v. A quantity of PEG 400 varying between 0% and 10% by weight with respect to the PCL content was added as a plasticizer. A variable amount of the solutions was poured into 9 cm diameter Petri dishes to obtain films with different thicknesses, placed in an oven at 60 °C for 2 days, and then left in the desiccator for at least 30 minutes before being removed from the supports.

4.3.1.3 Production of PCL supports by electrospinning

The solutions to be processed by electrospinning (Spinbow, Bologna, Italy) were prepared by dissolving the polycaprolactone, always at a fixed concentration of 20% w/v with respect to the solvent mixture volume, adding also in this case PEG 400 at different concentrations, from 0% to 10% w/w. The solutions were processed in variable quantities to obtain films with different thicknesses, using a voltage of 19 kV, a flow rate of 1.7 ml/h, a needle-collector distance of 17.5 cm and an 18-gauge needle.

4.3.1.4 Supercritical Fluids Impregnation (SFI)

The feasibility tests for the evaluation of the effect of the supercritical carbon dioxide ($scCO_2$) into contact with the PCL supports and the feasibility study of the loading of alpha-tocopherol into the polymer by supercritical impregnation were performed in the laboratory scale equipment described in section 2.2.

The contact tests with $scCO_2$ were carried out on the PCL samples produced by electrospinning and solvent casting. In particular, a fixed surface of each sample, of about 2 cm^2 at different thicknesses, was weighed and placed inside the stainless-steel autoclave. The tests were conducted at 35 °C and 170 bar investigating different contact times, from 15 minutes up to 72 hours. At the end of each test, after having depressurized the CO_2 to bring it back to environmental conditions, the surface of the samples and the weight were measured. Once the best combinations of samples in terms of thickness, percentage of PEG and contact time with $scCO_2$ had been identified, the study of supercritical impregnation of alpha-tocopherol was started. The impregnation process was conducted by placing a quantity of antioxidant inside the container mounted axially on the impeller, so that it came into homogeneous contact with the supercritical fluid once the process was started. The impregnation tests were carried out at different contact times to evaluate the impregnation kinetics.

4.3.1.5 Morphology characterization

The morphology of the samples was analyzed both before and after the supercritical process, using a Field Emission Scanning Electron Microscopy (FESEM, LEO 1525, Carl Zeiss SMT AG, Germany). All the samples were coated with a gold-palladium layer before being analyzed. The mean fibers diameter was evaluated using the ImageJ software, analyzing at least 300 fibers per sample.

4.3.1.6 Thermal analysis

The thermal analysis was conducted via Differential Scanning Calorimeter (DSC, TC11, Mettler-Toledo, USA), analyzing the samples both before and after the supercritical process to evaluate the effect of alpha-tocopherol on the polycaprolactone supports.

4.3.1.7 Alpha-tocopherol loading evaluation and migration tests

The effective loading of alpha-tocopherol on the polycaprolactone supports was evaluated by gravimetric method carried out on all the samples for each impregnation time tested. In particular, the samples were weighed before and after the SFI process and the real load was calculated as the difference between the final and the starting weight.

Finally, the migration tests of the alpha-tocopherol from the polymeric supports produced both by electrospinning and by solvent casting after the supercritical impregnation process were carried out. In detail, for each film, a known weight of sample was placed in contact with a defined volume of simulating fluid by immersion in static conditions. The food simulant chosen was ethanol at 50% v/v, to simulate the behavior of food products with lipid content. The tests were conducted at 40 °C until a constant release value was reached. The release liquids were analyzed by UV-vis spectroscopy at 295 nm at defined time intervals. The migration kinetics obtained were expressed as mg of alpha-tocopherol released compared to mg of alpha-tocopherol loaded by impregnation.

4.3.2 Results and Discussion

4.3.2.1 Feasibility study of the SFI process and PCL supports optimization

Solvent casting and electrospinning techniques were used to produce two different types of polymeric supports based on polycaprolactone, shown in Figure 78, to be used for the supercritical process. The films were made with different thicknesses to evaluate the influence of this parameter on the performance of the samples placed in contact with the $scCO_2$. To obtain different thicknesses, different volumes of solution were processed. In particular, through the solvent casting process, dense structures with a thickness between 50 and 200 μm were obtained by processing a volume of solution between 5 and 10 mL. Instead, films with a thickness between 30 and 300 μm and with a mean diameter of the fibers equal to 850 nm were obtained by means of electrospinning, processing from 5 to 10 mL of the PCL solution. The percentage of PEG, in the case of both techniques, was varied. The presence of PEG was particularly useful in the case of solvent casting, as it facilitates the removal of the samples from the Petri dishes.

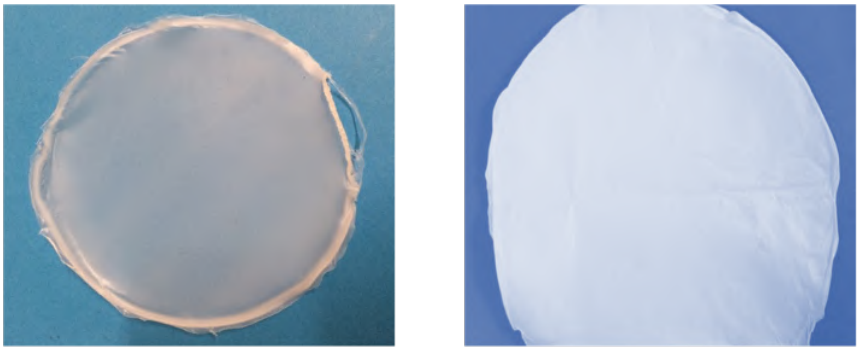


Figure 78: PCL 20% w/v films produced by (a) solvent casting and (b) electrospinning.

Once the films were obtained, the feasibility of the contact between these materials and the supercritical CO_2 was first assessed before loading alpha-tocopherol. This feasibility study has in fact shown that PCL, when placed in contact with $scCO_2$, is subject to a phenomenon of foaming that causes the formation of pores in the polymeric structure which can be a positive fact, as it can facilitate the impregnation of the desired compound. However, $scCO_2$ also causes a lowering of the melting temperature in semi-crystalline polymers. For this reason, optimizing the formulation of the solutions and the process operating conditions was necessary to obtain polymeric structures suitable for resisting against the contact with $scCO_2$. Campardelli et al., 2019 [266], observed that PCL is processable with $scCO_2$ at 40 °C and 150 bar or 35 °C and 200 bar. Starting from these data, the contact tests were carried out at 35 °C and 170 bar, varying both the thickness of the samples and the PEG content between 0 and 10% w/w with respect to the polymer content, and varying contact times between 15 minutes and 72 h.

The main results obtained from the contact tests between polymer and $scCO_2$ in terms of the macroscopic appearance of the post-process samples and evaluation of pore formation are summarized in Table 27.

Table 27:Contact tests with $scCO_2$ results. The test conditions were 35 °C and 170 bar at different contact time.

Thickness [µm]	% PEG	Contact time [h]	Macroscopic aspect	Formation of pores
		ELECTROSPINNING SUPPORTS		
< 100	0 – 10	15 min – 72 h	Non-intact structure	yes
> 100	0 – 5	< 2 h	Non-intact structure	yes
> 100	5 – 10	< 2 h	Intact structure	yes
		SOLVENT CASTING SUPPORTS		
< 100	0 – 10	15 min – 72 h	Non-intact structure	yes
> 100	0	< 24 h	Non-intact structure	yes
> 100	2.5 – 5	< 24 h	Semi-intact structure	yes
> 100	10	< 24 h	Intact structure	no

In particular, for the thinner samples produced by both techniques, with a thickness of less than 100 µm, the effect of melting temperature depression was predominant and the samples did not maintain the structure after contact with the $scCO_2$ for any time of contact investigated. Thanks to this study it was also possible to verify the importance of the presence of PEG in the samples which has been shown to act as a protective barrier against the polymer. In fact, the samples with PEG showed a better resistance against $scCO_2$, especially in the case of the supports produced by solvent casting which reached higher contact times than the electrospun samples. The SEM images carried out on the samples produced by solvent casting after contact with $scCO_2$ as a function of the PEG content, are shown in Figure 79. Instead, Figure 80 shows the SEM relative to the electrospun before and after the supercritical process carried out in the conditions identified as the best, in fact in the other cases it was not really possible to recover the material, as shown macroscopically in Figure 81.

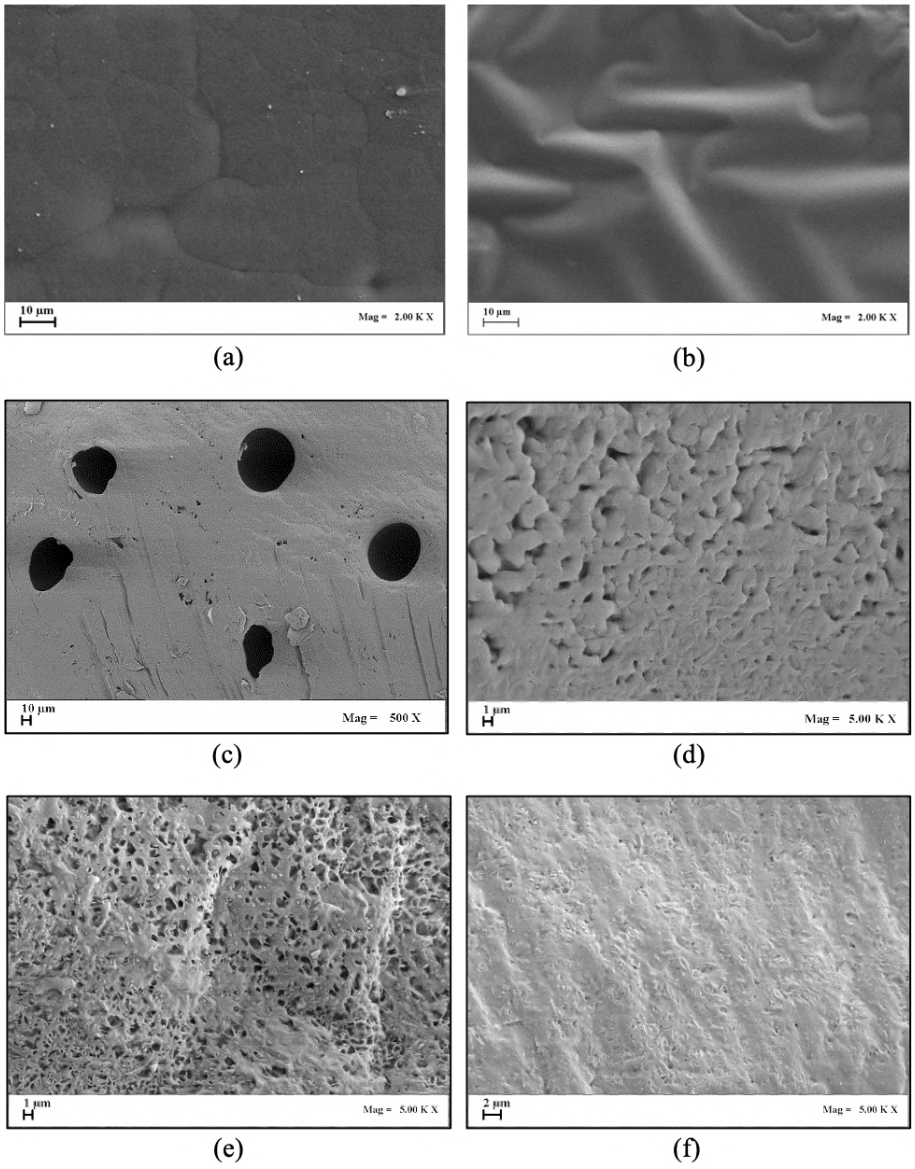


Figure 79: Effect of the PEG percentage on the structure of the samples obtained by casting before the $scCO_2$ contact (a) with PEG 2.5% w/w, (b) with PEG 10% w/w, and after the contact with $scCO_2$ for 24 h at 170 bar and 35 °C, (c) without PEG, (d) with PEG 2.5% w/w, (e) with PEG 5% w/w and (f) with PEG 10% w/w.

Figure 79 highlights the pore formation caused by the supercritical process. In particular, it is possible to note that the pore size is greater in the case of samples produced without PEG (Figure 79c), for which pores, with a mean size equal to 1.22 ± 0.28 μm, were obtained and then decreased as the content of PEG increased (Figure 79f), up to pores with a mean size of 0.15 ± 0.04 μm in the case of PEG at 10% w/w.

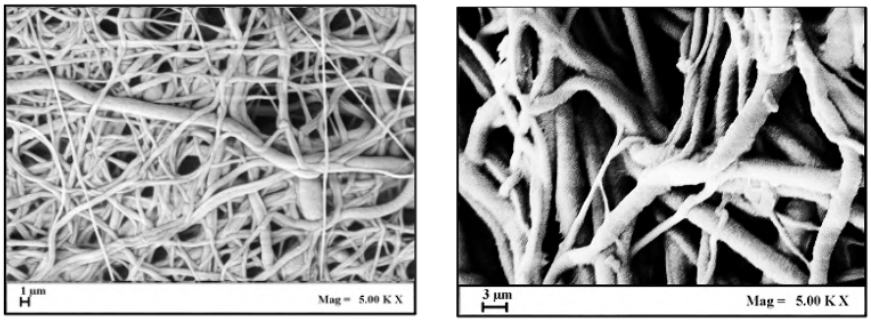


Figure 80: SEM images of the electrospun sample of PCL+PEG10% with a thickness of 500 µm, (a) before the contact with $scCO_2$ (b) and after the 2h of contact.

In the case of electrospun samples, from Figure 80 which reports SEM images of the electrospun fibers before and after the supercritical process at the same magnification, it is immediately possible to notice the swelling effect caused by the $scCO_2$ (Figure 80b), but without compromising the polymer structure. The conditions that therefore allowed to obtain an optimization of the supports, in terms of maintenance of the macrostructure (Figure 81b), were the following: sample thickness greater than 100 µm, with a PEG content equal to 10%, in particular for casting supports, and with a limit of contact times with the supercritical fluid equal to 2 h for the electrospun supports. For lower thicknesses, lower quantities of PEG, and higher contact times, the structure of the polycaprolactone is damaged as shown in Figure 81a.

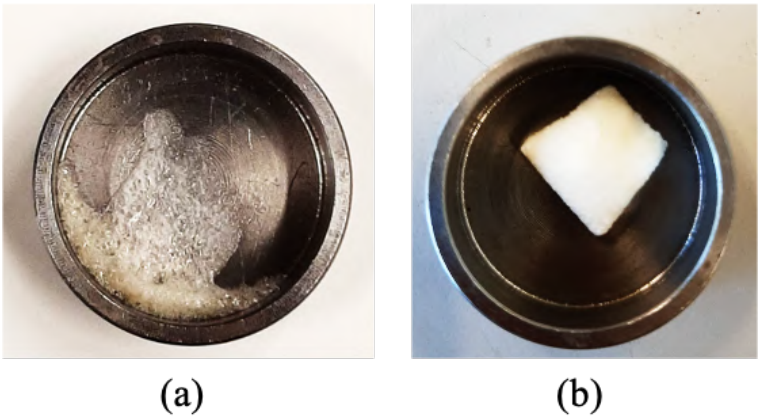


Figure 81: Appearance of electrospun PCL samples after contact with $scCO_2$:
(a) not optimized and (b) optimized.

4.3.2.2 Loading of alpha-tocopherol by SFI process

Once the optimal conditions for processing with supercritical fluids the polycaprolactone samples produced by electrospinning and solvent casting and also identified the relative limits, the study focused on the loading of alpha-tocopherol by supercritical impregnation. The impregnation with the SFI plant was carried out on samples with a size of 2 cm^2, using a temperature of 35 °C and a pressure of 170 bar, varying the impregnation times to investigate the kinetics. In particular, as regards the samples produced by solvent casting, it has been observed that the concentration of PEG is a determining parameter for the maintenance of the macrostructure; therefore, it was decided to initially set the process time at 24 h and to study the effect of impregnation as the PEG concentration varies between 0 and 10% w/w. The main results obtained are shown in Table 28.

Table 28: Supercritical impregnation of alpha-tocopherol in PCL films obtained by solvent casting varying PEG content, setting an impregnation time of 24 h at 35 °C and 170 bar.

PEG [%, w/w]	**alpha-tocopherol impregnated [mg_{TOC}/mg_{FILM}]**	**Effect**
0	1.234	Higher impregnation but significant modification of the structure
2.5	0.998	Impregnation and foaming
5	0.959	Impregnation and foaming
10	0.828	Lower impregnation but preservation of the structure

In particular, by loading the antioxidant into the PEG-free polycaprolactone supports, a greater effect of supercritical CO_2 was observed. In fact, as already observed in Figure 79c, larger pores were formed which allowed a greater loading of alpha-tocopherol but the macroscopic structure was damaged; in the case of the samples with a PEG content of 2.5% and 5%, both the tocopherol impregnation and the foaming effect were observed, while for PEG values equal to 10%, impregnation was found to be prevalent with respect to damage to the polymeric structure. Furthermore, as regards the alpha-tocopherol impregnation, lower loadings were obtained with increasing PEG percentage. Specifically, the load obtained went from 1.234 mg of tocopherol per mg of film in the case of PCL without PEG, up to 0.828 mg of tocopherol per mg of film loaded on PCL samples with PEG10% using the same contact time equal to 24 h. The morphological study, of which the SEM images are reported in Figure 82, can be of help in explaining this trend.

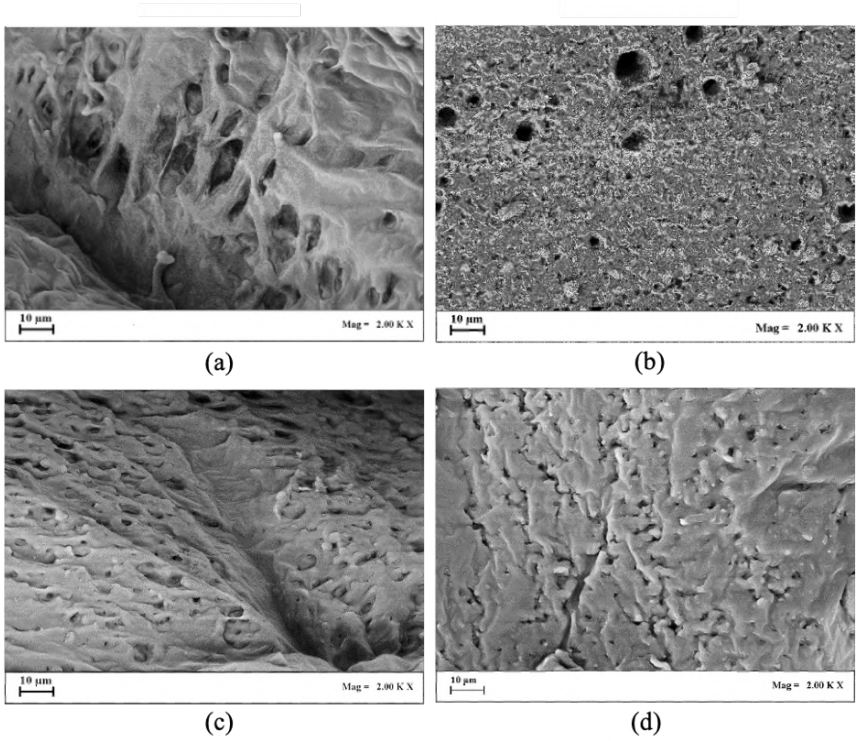


Figure 82: SEM images of the solvent casting samples at different PEG content after an impregnation time of 24 h, at 35 °C and 170 bar. (a) 0% PEG, (b) 2.5% PEG, (c) 5% PEG and (d) 10% PEG.

In fact, as had already been observed, as the concentration of PEG increases, the size of the pores decreases. Considering the closed-cell structures of the pores formed, the larger pores offer a greater exposed surface in which the alpha-tocopherol can be impregnated; consequently, the larger the pores, the greater the amount of alpha-tocopherol loaded.

In the case of samples obtained by electrospinning, the presence of PEG equal to 10% w/w has already been identified as a sine qua non condition for subjecting the samples to the supercritical process without damaging the structure for contact times of less than 2 hours. Therefore, the impregnation kinetics of alpha-tocopherol were studied, varying the impregnation time below this value. The tests were also conducted on the samples produced by solvent casting, using the same conditions, in order to be able to conduct a comparison between the impregnation kinetics. The results reported in the Table 29 show that the electrospun supports allowed a greater impregnation of the antioxidant than that of the samples produced by solvent casting with the same contact time. In fact, the quantity of alpha-tocopherol loaded into the fibers in just 2 h, was loaded onto the dense films in 15 h. The greater impregnation in the case of fibers is obviously due to the greater surface area exposed to contact with the supercritical CO_2 and therefore to contact with the active agent transported therein. Therefore, the porous structure of the electrospun sample, once optimized, can allow the same loading of active compound in a shorter time.

Table 29: Alpha-tocopherol impregnation in PCL+PEG10% films obtained by electrospinning and solvent casting by varying the impregnation time, at 35 °C and 170 bar.

Impregnation Time	**Alpha-tocopherol impregnated [mg_{TOC}/mg_{FILM}]**
PCL + PEG 10% ELECTROSPUN FILMS	
15 min	0.17
1 h	0.31
2 h	0.64
PCL + PEG 10% CASTED FILMS	
2 h	0.25
6 h	0.40
15 h	0.67
24 h	0.82

In order to verify if the experimental and theoretical data were in good agreement, the kinetic data were fitted with pseudo-first-order and pseudo-second-order equations, which were obtained by integrating the following equations 13 and 14 respectively [240]:

$$dq_t/dt = k_1(q_e - q_t) \quad (13)$$

$$d\left(1 - \frac{q_t}{q_e}\right)/dt = -k_2\left(1 - q_t/q_e\right)^n \quad (14)$$

Where, q_e is the adsorption capacity at equilibrium, i.e., the maximum amount of alpha-tocopherol impregnated into the PCL-based support (mmol/g), while q_t (mmol/g) is the adsorption capacity after the contact time "t" (h), k_1 is the pseudo-first order rate constant (1/h) and k_2 is the pseudo-second-order rate constant (g/mmol h). These equations were expressed by its integration and linearization as reported in equation 15 and 16 respectively [240]:

$$\log(q_e - q_t) = \log(q_e) - k_1/2.303\, t \quad (15)$$

$$t/q_t = t/q_e + 1/k_2 q_e{}^2 \quad (16)$$

A comparison between the impregnation rate constants obtained from the models is reported in Table 30, from which it was possible to observe that the pseudo-first-order kinetics fitted better the experimental data compared to the pseudo-second-order kinetics comparing the R^2 values obtained. Plotting $-\log(q_e - q_t) + \log(q_e)$ versus time, k_1 was determined by the slope of the fitting, as reported in Figure 83.

Table 30:Impregnation rate constant obtained from the pseudo-first-order and pseudo-second-order kinetics for electrospun and casted PCL+PEG10% supports impregnated at 35 °C.

Support	Pseudo-first-order kinetics			Pseudo-second-order kinetics		
	q_e, mmol/g	k_1, 1/h	R^2	q_e, mmol/g	k_2, g/mmol h	R^2
Electrospun	1.486	0.696	0.964	2.094	0.117	0.714
Casted	1.904	0.114	0.994	1.611	0.543	0.922

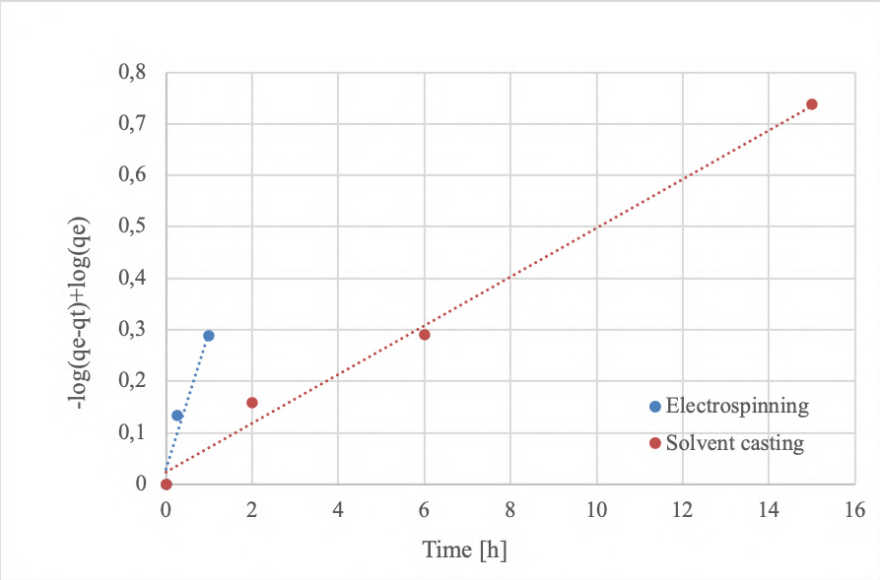


Figure 83: Pseudo-first-order kinetics for the supercritical impregnation of alpha-tocopherol into electrospun and casted PCL+PEG10% samples at 35 °C and 170 bar.

At this point it is interesting to observe the morphological aspect of the fibers after supercritical impregnation at different contact times, shown in the SEM images of Figure 84. In particular, it is possible to note the effective plasticizing action of alpha-tocopherol which contributes to lowering the melting temperature of the polymer, even more than $scCO_2$ alone [267,268]. In fact, the fibers

obtained after impregnation with alpha-tocopherol for 2 h (Figure 84c) have an irregular and slightly melted appearance compared to those obtained through the contact of the polymer with only $scCO_2$ (Figure 80b). Instead, the fibers obtained for impregnation times equal to 1 hour and 15 minutes have maintained a more regular structure. Furthermore, observing Figure 84a, relating to an impregnation time of 15 minutes, it is possible to observe the presence of alpha-tocopherol in the form of nanoparticles arranged on and inside the fibers. In fact, during the depressurization phase, the carbon dioxide, with the active ingredient solubilized in it, returns to the gaseous form and therefore, the solute precipitates.

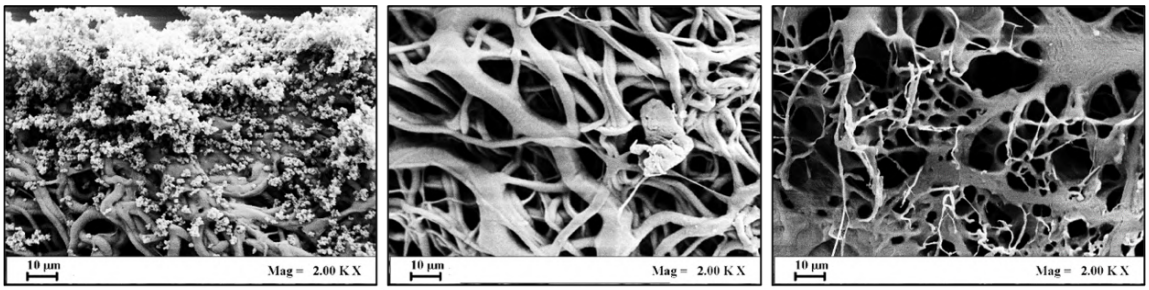


Figure 84: SEM images of the electrospun fibers impregnated with alpha-tocopherol at 35 °C and 170 bar at different impregnation times: (a) 15 min, (b) 1h, and (c) 2h.

Furthermore, to investigate the plasticizing effect that the samples produced by solvent casting have been shown to undergo and to investigate the difficulty of preserving the polymeric structure, it was interesting to conduct a thermal analysis, whose thermograms are reported in Figure 85. The tests were conducted on the samples at different PEG concentrations and for alpha-tocopherol impregnation times of 24 h. For the samples produced by electrospinning, the thermal behavior of the foamed films was not evaluated as it was not possible to recover the material for other analyzes. By comparing the DSC thermograms of polycaprolactone films before the supercritical process and after the foaming effect induced by the supercritical process, the plasticizing effect of $scCO_2$ alone, which lowers the melting temperature of the polymer, was evident. Then comparing the films subjected to supercritical impregnation of alpha-tocopherol, it is clear that this also acts as a plasticizer for the polymer, therefore it further anticipates the melting temperature; hence the initial difficulty in maintaining the structure of the film.

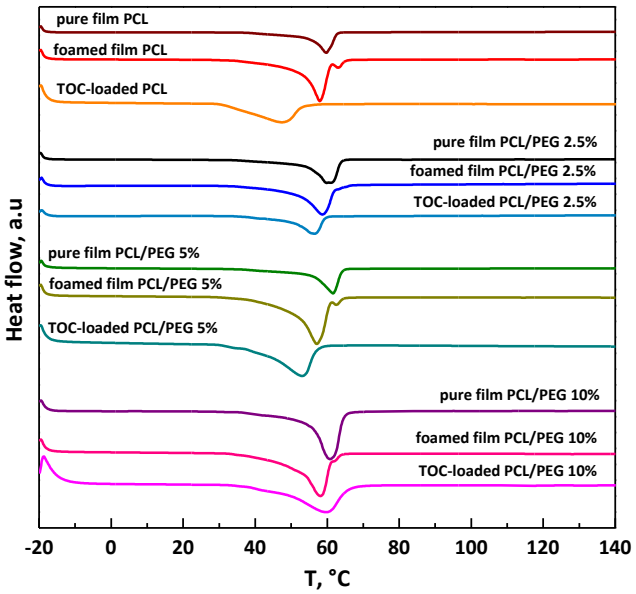


Figure 85: DSC thermograms of pure casted films and foamed films in contact with $scCO_2$.

4.3.2.3 Migration test of alpha-tocopherol in food simulant

Finally, the migration tests of alpha-tocopherol from the optimized polymeric supports were carried out, considering, for each technique, the maximum reachable impregnation time, and therefore the maximum load. In particular, PCL samples with 10% PEG produced by electrospinning and impregnated for 2 h and PCL samples with 10% PEG produced by casting and impregnated for 24 h were analyzed. The tests were conducted by immersion method in static at 40 °C, using a hydroalcoholic solution of ethanol 50% v/v as simulant fluid for food products with lipid content. The release fluids were analyzed by UV-vis analysis at 295 nm at regular time intervals for one week. The results were expressed as a percentage of mg of alpha-tocopherol released per mg of theoretically impregnated alpha-tocopherol. The quantity of sample placed in contact with the simulant was evaluated in order to have the same initial theoretical load of impregnated alpha-tocopherol, i.e., 9.5 mg per sample for both techniques in order to be able to carry out a comparison, reported in Figure 86.

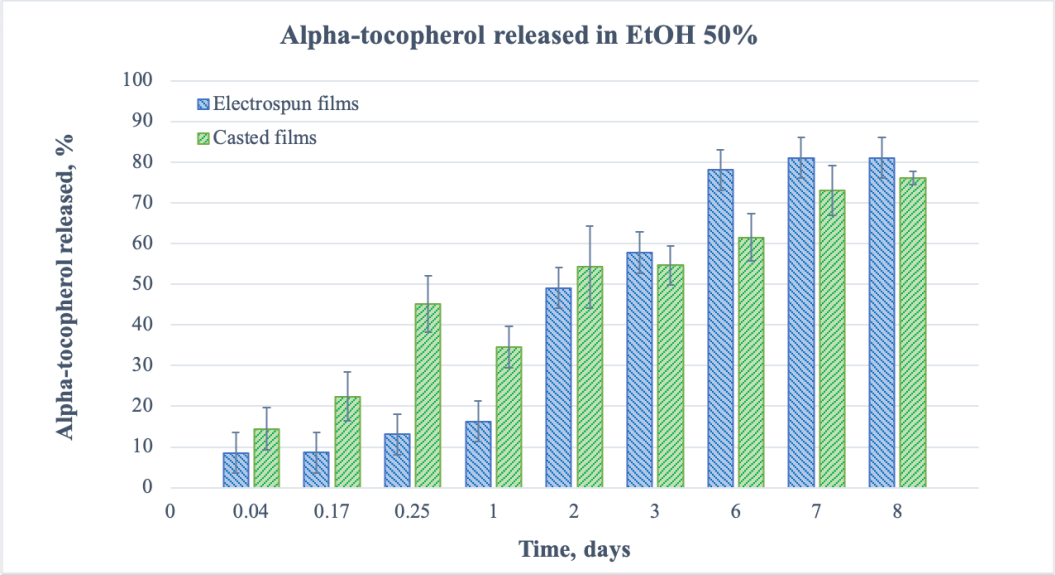


Figure 86: Releases of alpha-tocopherol from electrospun sample impregnated for 2 h and from casted sample impregnated for 24 h, in EtOH 50% v/v at 40 °C for a week.

Unlike the migration tests conducted on the samples of the previous chapter dedicated to zein, the polycaprolactone samples impregnated with supercritical fluids showed different behavior. In fact, the films produced by solvent casting showed a higher release kinetics in the first hours of the test compared to the electrospun samples, although the latter have a much greater surface exposed to the simulant due to the fibrous nature. Only after two days, the migration kinetics from the electrospun samples becomes greater than those produced by casting. This trend is due to the fact that alfa-tocopherol penetrated much better into the fibers for contact times of 2 h, compared to casting films impregnated for 24 h, where instead the impregnation was more superficial, as the pores for PCL with PEG at 10% w/w was small with a smaller surface area exposed to the supercritical process. For this reason, the release from the fibers is more gradual and constant over time. For both techniques, however the half of the impregnated alfa-tocopherol was released in just 48 h.

To investigate the release mechanism, these new experimental data were studied using kinetic models available in literature. In fact, the possible mechanisms through which a release occurs can be associated, in addition to the concentration gradient between sample and bulk, to diffusion phenomena, but also to swelling or erosions and degradations of the polymeric matrices with consequent diffusion of the compound loaded, or they may be associated with a combination of these mechanisms. The zero-order release models were not considered as they are applicable only in the case of very slow-release kinetics. The first-order release models describe concentration-dependent release kinetics, but in this case, having never investigated which phenomenon is associated with the

releases obtained, the exponential model of Korsmeyerñ-Peppas [269] or Power Law, and the Gallagher-Corrigan model [270] were used and compared, being applicable precisely in the case in which the release mechanisms are not known or in the case in which several phenomena can contribute to it. The equations used in these models are reported in equation (17), (18) and (19) respectively:

$$M_t/M_\infty = k_m t^n \tag{17}$$

In the Korsmeyerñ-Peppas equation (17), M_t is the amount (mg) of substance released at time "t", M_∞ is the total amount (mg) of substance that can be released at infinite time, k_m is the release constant and n is a release exponent estimated from linear regression of $\log(\frac{M_t}{M_\infty})$ versus log(t) which indicates the release mechanism. If n=0.45, the mechanism of release is fickian diffusion, if 0.45<n<0.89, it indicates that the mechanism is not fickian diffusion but a combination of both diffusion and erosion-controlled rate release.

$$F_{TOT} = F_B(1 - e^{-K_B t}) + (1 - F_B)\left(\frac{e^{kt-kT_{max}}}{1+e^{kt-kT_{max}}}\right) \tag{18}$$

In the Gallagher-Corrigan equation (18), F_{TOT} represents the total fraction of substance released at time "t" and it is given by the sum of two contributions, the surface diffusion and the degradation released mechanism. F_B is the fraction of substance available for direct surface release, dependent on the geometry of the film and on the geometry and sizes of substance particles; K_B is the release rate constant dependent on the diffusion coefficient, solubility of the substance, the surface exposed to the simulant as reported in [270]. The second contribution is related to a release controlled by polymer degradation mechanism where, k is the rate constant and T_{max} is the time for which the maximum release rate is expected.

A comparison among the release rate constant and diffusion coefficient that better fitted each model are reported in Table 31.

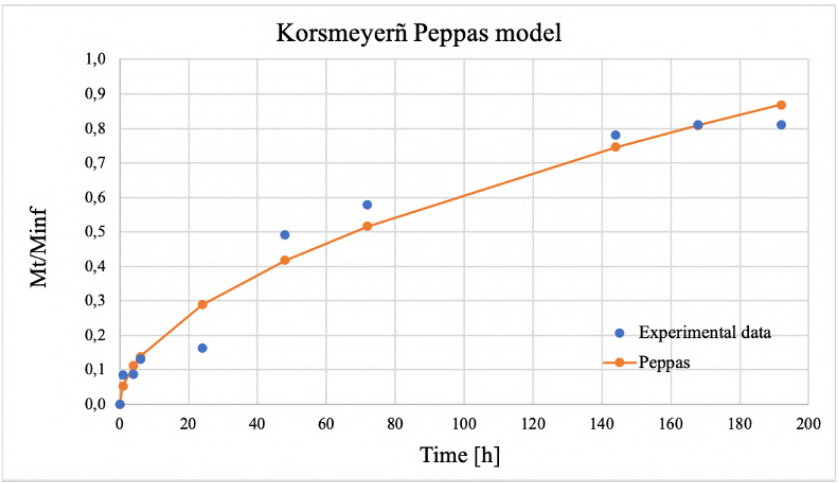
Table 31: Release rate constant and diffusion coefficients obtained from the Korsmeyerñ-Peppas and Corrigan Models for electrospun and casted PCL samples impregnated with alpha-tocopherol via $scCO_2$ process.

Parameters	Electrospinning	Solvent casting
Korsmeyerñ-Peppas Release Model		
k_m (-)	0.053	0.188
n (-)	0.529	0.256
R^2	0.984	0.964
Gallagher-Corrigan Release Model		
K_B (day^{-1})	0.302	5.952
F_B (-)	0.894	0.393
k (day^{-1})	0.101	0.382
T_{MAX} (day)	30	7
R^2	0.991	0.970

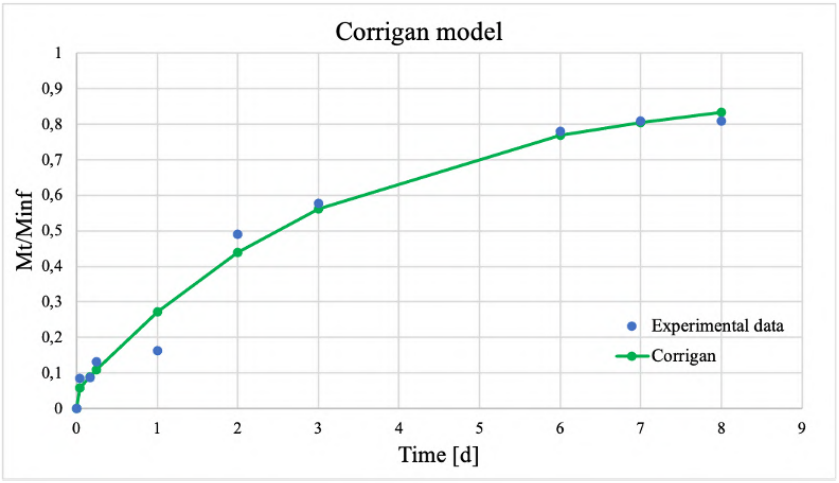
The curves obtained from model fitting studies are reported in Figure 87 related to the impregnated electrospun samples and in Figure 88 related to the impregnated samples produced by solvent casting. Comparing the values of the coefficient of determination R^2 reported in Table 31, it was found that both models fit with the experimental data.

In particular, the "n" exponent value obtained from Peppas model, in the case of the electrospun samples was indicative of a non-fickian transport highlighting both diffusion and probably swelling phenomenon of the matrix due to the fibrous structure, while in the case of the samples produced by solvent casting it has indicated a quasi-fickian diffusion due to the non-swellable matrix diffusion as described by Paarakh et al., 2019 [271]. While, from the Corrigan model it emerged that for the release times investigated, the beginning of the polymer degradation was not reached since the PCL has a much longer degradation time than that of the test. Furthermore, in the case of electrospun fibers, the release is due to diffusion phenomena of the antioxidant available on the surface while the amount encapsulated within the fibers (about 19% from experimental data) will probably be released when the polymer degrades. In the case of solvent casting, on the other hand, it was possible to observe a biphasic release, with an initial rapid release with first-order kinetics, burst phase, due to the rapid dissolution of the alpha-tocopherol present on the surface of the films, as also observed during the release tests, followed by a lag period without reaching PCL degradation.

The difference in the behavior of the two supports is also highlighted by the K_B values, so the higher values were obtained in the case of the solvent casting samples.

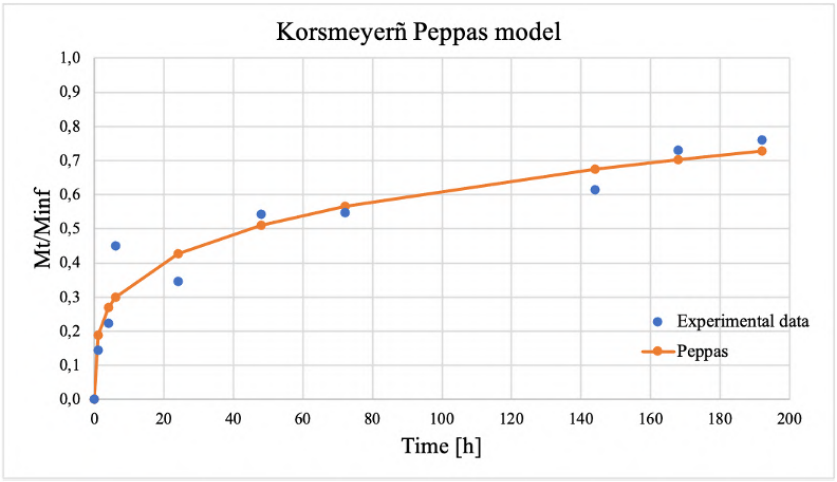


(a)

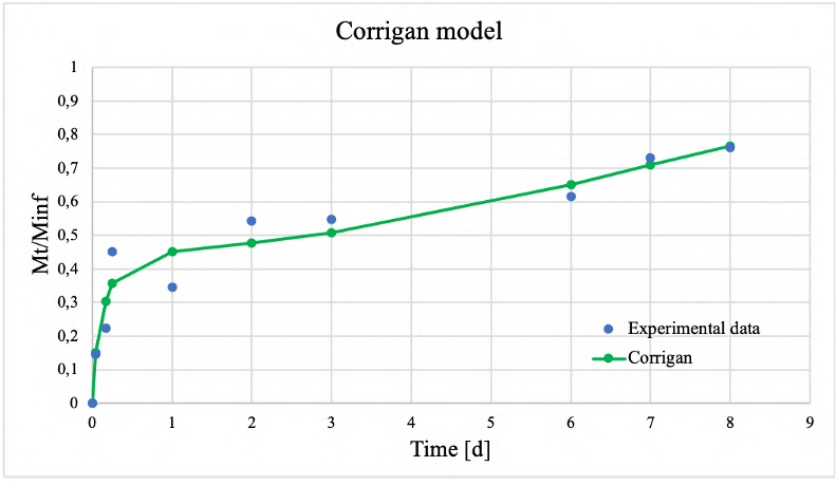


(b)

Figure 87: (a) Korsmeyerñ-Peppas kinetic release model and (b) Corrigan model for alpha-tocopherol release from supercritical fluids impregnated electrospun PCL samples.



(a)



(b)

Figure 88: (a) Korsmeyerñ-Peppas kinetic release model and (b) Corrigan model for alpha-tocopherol release from supercritical fluids impregnated casted PCL samples.

In conclusion, this section allowed to successfully test a method of loading active compounds into polymer matrices after sample production, as is usually done on an industrial scale using green techniques. In fact, the supercritical impregnation technique offers a valid alternative being a green technique that can work with fluids such as CO_2 that allows supercritical conditions to be reached by operating at mild temperature and pressure values. Furthermore, despite the difficulty of the polycaprolactone process through this technique, the optimal conditions have been identified to be able to subject two different types of polymeric structures to the impregnation of active compounds. The films obtained have therefore proved to be potentially useful for applications on foods with a lipid content given the results of the migration tests, offering a valid alternative to synthetic but non-biodegradable polymers.

5 ELECTROSPINNING ECONOMIC AND FINANCIAL ANALYSIS

PURPOSE

The electrospinning used in this work is a laboratory-scale equipment for the production of polymeric materials composed of nano and micrometric fibers. Electrospinning has been recognized as a particularly versatile technique in the manufacture of polymeric supports with the possibility of encapsulating molecules with high-added value due to the characteristic mild conditions of the process, useful for applications in various fields, from biomedical and pharmaceutical, to the textile and membranes for purification, and finally, useful in the field of food packaging. While in other sectors this technique is already used on an industrial scale, in the field of food packaging it is today a technique mainly used on a laboratory scale but of interest to many researchers.

A business plan for the commercialization of electrospun polymer films for active food packaging products was attempted to investigate if the processing of these products using electrospinning could be profitable.

5.1 Economic analysis

To discuss this chapter, it is necessary first of all to consider the commitment of governments worldwide in addressing the reduction of environmental pollution to which, as mentioned in the "Introduction" chapter, the packaging sector contributes enormously due to the use of disposable non-degradable packaging. The goal is to opt for eco-friendly solutions. This goal is so strong that the European Union has set 2025 as the deadline for achieving "zero plastic waste" in landfills. The solution to meet this objective is to be found in the use of eco-sustainable materials, which meet the requirement of the three "R", to renew, reuse and recycle. From this point of view, biopolymers are excellent candidates.

To assess whether it is worth investing in the field of food packaging, it is sufficient to observe that the size of the market for this sector closed, in 2021, at 49.8 billion dollars with a growth forecast of 6.4% to reach 72.3 billion of dollars by 2027 (https://www.marketsandmarkets.com/Market-Reports/food-packaging-films-market-155846613.html). This is because packaging plays a fundamental role in food products, both from the point of view of quality and safety and marketing.

Most of the costs associated with the production of film for food packaging are due to the raw materials. The price of the fossil-based polymers used depends on the price of oil. Considering the fluctuating trend in the price of oil, especially in the current historical period, it is foreseeable that the price of plastics will also be destined to rise. In reality, this upward trend has already begun.

The size of the biopolymer film market was instead equal to about 4 billion dollars in 2021 with a growth forecast of 8.4% by 2028 precisely due to the growing demand for sustainable packaging (https://www.gminsights.com/industry-analysis/biopolymer-films-market).
Growth in the food sector and the growing demand for packaging products from renewable sources for prolonged food storage have driven the biopolymer film market in recent years. The abundant availability of bio-based materials including proteins and polysaccharides will likely push the value of the biopolymer film market beyond the forecast period.

5.2 B2B Model

To study the profitability of the production of zein-based polymeric films enriched with vanillin as an antimicrobial agent by electrospinning to be used as patches to be inserted inside the food packages of fresh products to satisfy the request of a hypothetical food company, a B2B model was used by which the CAPEX (Capital Expenditure) and OPEX (Operating Expenditure) were estimated to produce a hypothetical 5-year prevision (2023-2027) for commercialization. The high versatility of the electrospinning process has already been recognized in terms of process sustainability, food compatibility and applicability in various industrial sectors.
The gain of this process is referred to the one-shot production of packaging materials with an automatized, continuous and reproducible plant layout. By now, the electrospinning equipment, analyzed in this work, has a Technology Readiness Level (TRL) of 3 (Figure 89).

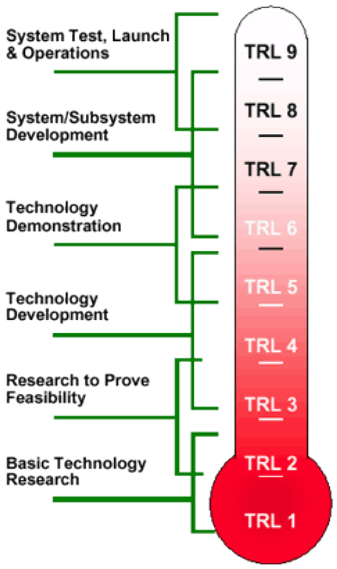


Figure 89: Technology Readiness Level (https://en.wikipedia.org/wiki/Technology_readiness_level).

But in reality, considering that there is already industrial-scale equipment used today in other fields other than packaging (for example, YFlow SD, Elmarco reported in Figure 90), the project to carry out this industrial process should be easily realized. Therefore, to conduct this economic study, it was decided to use the TRL equal to 7, considering starting from a prototype dedicated to the production of packaging materials.

Figure 90: Example of electrospinning system on industrial scale. Nanospider industrial electrospinning technology of Elmarco (https://www.elmarco.com; http://electrospintech.com/mass-production.html#.Y00hzC0QNAY).

5.3 Financial Analysis

The financial analysis for the commercialization of biodegradable antimicrobial electrospun patches for packaging, needs to be divided into CAPEX and OPEX. CAPEX is linked to the setting up of the electrospinning plant, but also for desks, computers and mobile phones. CAPEX amortization was fixed as linear, with a yearly depreciation of 10% over a period of about 10 years.

Productivity was fixed at 550 m^2 of films per day and 300 working days per year.

The selling price was set at about 9 euro/m^2, equal to about 8 cents per patch. The selling price was evaluated considering the retail prices of food products such as red meat, fish, red fruits, etc., for which the patch produced would reasonably weigh about 2% of the total price of the packaged product.

The CAPEX cost of electrospinning is reported in Table 32 and represents the value of the assets acquired by the company whose duration is greater than the fiscal year.

Table 32: CAPEX cost for biodegradable electrospun packaging materials.

ASSET DESCRIPTION	unit qty	cost/unit [€]	TOTAL COST [€]
Electrospinning machine	5	60000	300000
Solution preparation system (tanks, pumps and flowmeters)	1	5000	5000
Solution viscosity measure system	1	1500	1500
Solution feeding system - pump (0.5 KW, Qmax = 900 L/h)	1	450	450
Solution feeding system - valves (solenoid 3/8")	5	50	250
Solution feeding system - piping (PTFE piping 1/2", 3/8")	1	11000	11000
Components up-grade (software and hardware)	1	35000	35000
Desks and chairs	6	300	1800
Drawers	3	80	240
Personal Computer	3	1000	3000
Automation and Server	1	11000	11000
Company cars	1	20000	20000
Mobile phones - management	2	800	1600
Mobile phones - employees	4	350	1400
TOTAL ASSETS COST [€]			**392240**

The OPEX are reported in the following Tables 33-36. The total marketing costs are given by the sum of the expenses made annually for events and fairs, communication and promotion strategies via the web.

Table 33: Marketing cost.

MARKETING COSTS	2023	2024	2025	2026	2027
# of Events & fairs	1	1	2	2	3
Fee	3000	3000	6000	6000	9000
Stand Preparation	7500	8000	8500	9000	9500
Flights, hotels, meals	9600	9600	19200	19200	28800
other costs	960	960	1920	1920	2880
EVENTS & FAIRS	21060	21560	35620	36120	50180
Advertising %Δ	0%	2%	4%	6%	8%
Magazines	500	510	530	562	607
TV & Radio	2500	2550	2652	2811	3036
Digital Ads	6000	6120	6365	6747	7286
Brochure & gadgets	1500	2000	3000	3500	4000
Professional Video	2000	2000	4000	4000	6000
Sponsorship	2000	2000	4000	4000	6000
COMMUNICATION	14500	15180	20547	21620	26930
Costs %Δ	0%	1%	2%	3%	4%
Social media strategy	2400	2424	2472	2547	2649
WEB	2400	2424	2472	2547	2649
MARKETING TOTAL COSTS [€]	**37960**	**39164**	**58640**	**60287**	**79758**

Table 34: Subcontractors costs.

SUBCONTRACTORS	2023	2024	2025	2026	2027
	0%	8%	5%	5%	3%
Project & management	0	40000	2000	0	0
Freight of plant element	0	5000	2500	0	0
Automation & integration costs	0	10000	9000	4000	4000
Financial Advisor	3000	3240	3402	3572	3679
Legal expenses	3000	3000	2500	1500	1500
SUBCONTRACTOR [€]	**6000**	**61240**	**19402**	**9072**	**9179**

In the first year, all the dynamics are foreseen for the definition of the machinery installation contracts and the start-up costs of the company, the second and third years involve transport and installation costs of the system (which occurred in the last months of the first) and the cost to be considered for the management of the plant automation. While from the fourth year only the costs related to the maintenance upgrade and current management were considered.

Table 35: Operating and production costs.

OPERATING	QTY	COST	Notes	2023	2024	2025	2026	2027
Costs %Δ				0%	8%	5%	5%	3%
Mobile phones	6	120	# of employee	720	778	816	857	883
Power & water supply	1	10000	yearly	10000	10800	11340	11907	12264
Internet	12	100	montly	1200	1296	1361	1429	1472
Bank account	12	5	montly	60	65	68	71	74
PEC	12	30	montly	360	389	408	429	442
Insurance	1	4000	yearly	4000	4320	4536	4763	4906
Software licence	12	500	montly	6000	6480	6804	7144	7359
Rent	1	5000	yearly	5000	5400	5670	5954	6132
Mailing services	12	1000	montly	12000	12960	13608	14288	14717
Polymers (Zein) [kg]	6336	45.42	yearly	0	287781	302170	317279	326797
Solvents								
Deionized water [L]	6120	1.6	yearly	0	9792	10282	10796	11120
Ethanol absolute [L]	24336	16.48	yearly	0	401057	421110	442166	455431
Additive (Vanillin) [kg]	350	127.4	yearly	0	44545	46773	49111	50585
Maintenance	1	10%		3934	4249	4461	4684	4825
OPERATING				**43274**	**789912**	**829407**	**870878**	**897004**
Costs %Δ				0%	5%	10%	15%	20%
Accounting, tax & legal	1	3000	yearly	3000	3150	3465	3985	4782
Business & strategy	1	3500	yearly	3500	3675	4043	4649	5579
Other services	1	10%	% (other cost lines)	650	683	751	863	1036
SERVICES				**7150**	**7508**	**8258**	**9497**	**11396**
OPERATING TOTAL COSTS [€]				**50424**	**797419**	**837666**	**880375**	**908400**

The first part of the table devoted to operating costs was increased based on forecast inflation. Service costs were increased based on an increase in turnover.

Table 36: Personnel costs.

ROLE	COST 2023	%ETF 2023	%ETF 2024	% ETF 2025-2027	2023	2024	2025	2026	2027
					0%	3%	2%	2%	3%
CEO	80000	60%	60%	60%	48000	49440	50429	51437	52980
Project Manager	35000	100%	100%	100%	35000	36050	36771	37506	38632
Operator	28800	20%	100%	100%	5760	29664	30257	30862	31788
Operator	28800	0%	100%	100%	0	29664	30257	30862	31788
Adminis-trative	30000	100%	100%	100%	30000	30900	31518	32148	33113
PERSONNEL [€]					**118760**	**175718**	**179232**	**182817**	**188302**

The first year of investments will be used to obtain customer contracts and installation of the production plant. By the second year, the steady state production and sale of products will be obtained. By the end of third year, it will be possible to start to invest again to improve the production.

In the following Tables 37 and 38, are reported the Profit and Loss Statement and a Cash Flow Statement.

Table 37: Profit and Loss Statement.

PROFIT & LOSS	2023	2024	2025	2026	2027
Film produced [m^2]	0	165000	165000	165000	165000
sell price/m^2	0	9	11	12	13
Film sold [m^2]	0	156750	156750	156750	156750
Film sold	0	1410750	1724250	1881000	2037750
TOTAL REVENUE	**0**	**1410750**	**1724250**	**1881000**	**2037750**
TOTAL COGS	0	706017	741318	778384	801735
COST OF NOT SOLD	0	37159	39017	40968	42197
GROSS MARGIN	**0**	**667574**	**943915**	**1061649**	**1193818**
GM %	**0.0%**	**47.3%**	**54.7%**	**56.4%**	**58.6%**
PERSONNEL	118760	175718	179232	182817	188302
EVENTS & FAIRS	21060	21560	35620	36120	50180
COMMUNICATION	14500	15180	20547	21620	26930
WEB	2400	2424	2472	2547	2649
MARKETING	37960	39164	58640	60287	79758
OPERATING	50424	54243	57331	61023	64469
SERVICES	7150	7508	8258	9497	11396
SUBCONTRACTOR	6000	61240	19402	9072.1	9179.2
LTD	33550	35750	37950	40150	42350
TOTAL COSTS	**253844**	**373623**	**360813**	**362846**	**395454**
EBITDA	**-253844**	**293951**	**583102**	**698803**	**798364**
AMORTIZATION	35724	39224	39224	39224	39224
DEPRECIATION	0	3138	3138	3138	3138
EBIT	**-289568**	**251589**	**540740**	**656441**	**756002**
INTEREST	21450	19250	17050	14850	12650
EBT	**-311018**	**232339**	**523690**	**641591**	**743352**
Taxes	0	85966	193765	237389	275040
NET PROFIT [€]	**-311018**	**146374**	**329925**	**404202**	**468312**
NP %	**0.00%**	**10.38%**	**19.13%**	**21.49%**	**22.98%**

The films actually sold were considered 95% of the annual total produced. The total cost of goods sold (COGS) was assessed on the basis of the operating conditions (mainly the quantity of raw materials) investigated in this Ph.D. work. LTD (Long term debt) is the principal amount of the loan repayment, the annual increase is due to the French amortization used to calculate the loan, which considers a constant monthly payment.

The EBITDA "Earning Before Interest Taxes Depreciation and Amortization" represents the gain before considering the items listed.

Table 38: Cash Flow Statement.

CASH FLOW	2023	2024	2025	2026	2027
EBITDA	-253844	293951	583102	698803	798364
Δ INVENTORY	0	14120	14826	15568	16035
Δ TAXES	0	85966	193765	237389	275040
CFO: OPERATING CASH FLOW	-253844	193865	374510	445846	507289
CFI: INVESTING CASH FLOW	-392240	0	0	0	0
PROVISION	3904	39224	39224	39224	39224
LONG TERM DEBT	550000	0	0	0	0
EQUITY INJECTION	75000	0	0	0	0
INTEREST	21450	19250	17050	14850	12650
CFF: FINANCING CASH FLOW	650354	58474	56274	54074	51874
CASH FLOW = CFO + CFI + CFF	**4270**	**252339**	**430784**	**499920**	**559163**

By the previous Tables it was possible to obtain the following diagrams reported in Figures 91, 92 and 93.

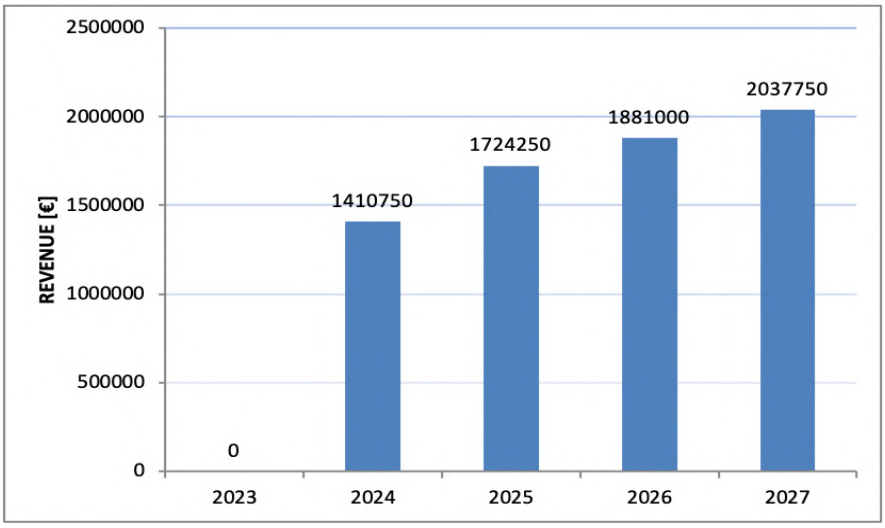


Figure 91: Revenues calculated for electrospun packaging materials commercialization (2023-2027).

During the first year of exercise, the aim will be the set-up of the production plant and the statutory requirements. During the second year, it is expected to have 100% of possible production with a Revenues for 1.4 million € assuming a sell price of 9 € per m^2, this price is equivalent to 8.6 cents/patch for high-density food product with a typically price of 3-4 € such as red fruits or red meat package products.

In subsequent years, taking advantage of the regulations against plastic, it will be possible to increase the sale price to 13 € per m^2, reaching 2027 with a turnover of 2 million €, also taking into account the increase in the costs of raw materials and labor.

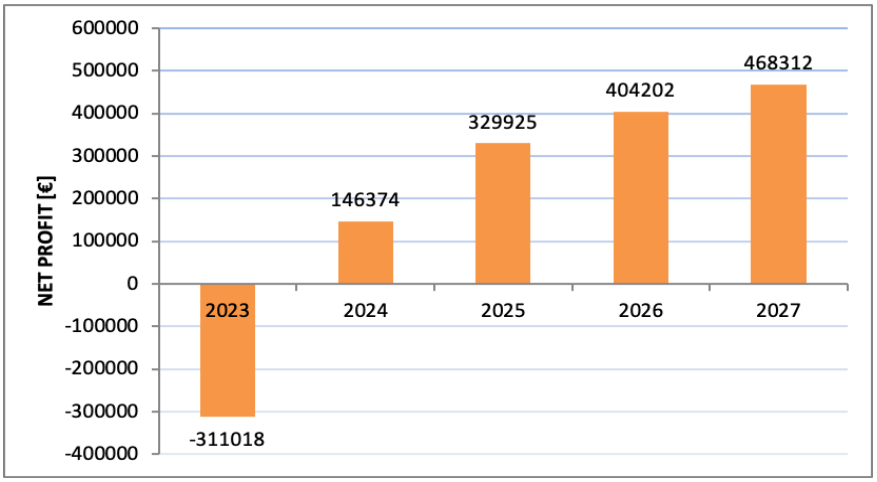


Figure 92: Net Profit diagram for electrospun packaging materials commercialization (2023-2027).

The Net Profit graph shows that the first year the profit will be negative, as there are no sales but only investments and installations. From the second year, the net profit will be positive (representing 30% of the debt) with a forecast of being able to reinvest in the third year to increase production and optimize structural costs.

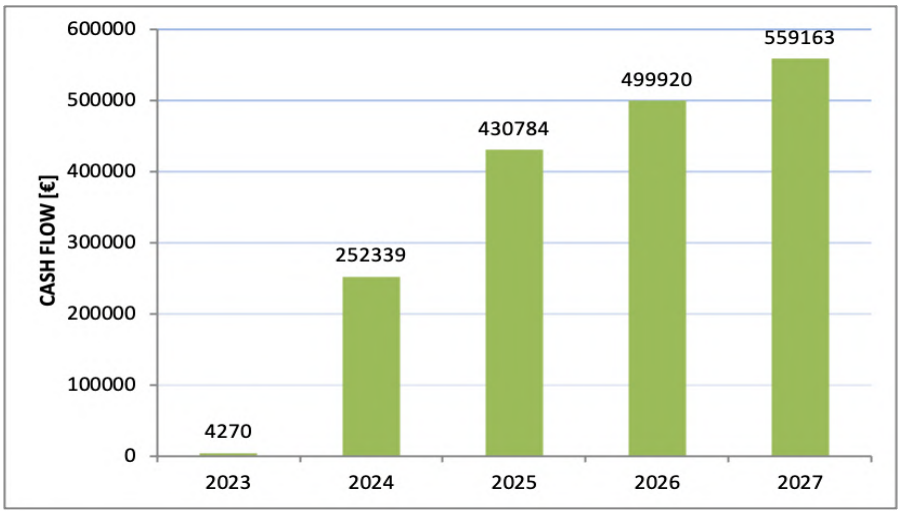


Figure 93: Cash Flow Statement for electrospun packaging materials commercialization (2023-2027).

An initial loan of 550.000 € was assumed to generate the Cash Flow graph.

In conclusion, the electrospinning technique for the production of antimicrobial films to be inserted inside the primary fruit and meat packages can be profitable if it is possible, on the basis of the market, to reach the sales price identified as appropriate in this analysis, 9-13 €/m^2.
In particular, the reference product will be antimicrobial patches to be inserted inside packages of fresh fruit and/or meat with dimensions of about 12 cm x 8 cm, which will be marketed at about 8 cents per piece. This price was comparable to that of biodegradable trays for food, sold at 8 cents per piece, and was lower than that of waxed paper disks for meat products sold for up to 40 cents per piece, while it is currently difficult to find information on bio-based films for food packaging, as they are still not very present on the market. Obviously, for the current market situation, the price per square meter of polymeric films of fossil origin remains much lower (about 3 €/m^2 for plastics antimicrobial films), although it is destined to grow in the coming years. The factor that raises the price of natural films is mainly attributable to the price of raw materials, in this case, zein and vanillin, so that, in order for products of this type to become competitive, the intervention of legislators will be necessary for the definition of incentives aimed at transition to zero plastics wastes.

6 GENERAL CONCLUSIONS

In this study, the potential use of biopolymers and natural compounds for the production of materials for active food packaging realized using more ecological techniques than conventional ones and more suitable for the treatment of these materials was investigated, especially in terms of the use of solvents and high temperatures. In fact, only non-toxic solvents recognized as GRAS were used in this work, while the techniques adopted did not involve the use of high temperatures. The treated materials were mainly two, zein and polycaprolactone. While polycaprolactone is a polymer that has already been extensively investigated in other fields, especially in the biomedical one for its compatibility, biodegradability, and good workability properties, zein instead has aroused scientific interest only more recently in the field of materials, since, due to its low amino acid profile, it is not a protein of interest for nutrition and, therefore, its revaluation through application in the world of food packaging is of great interest in a circular economy perspective. The interest in using only biodegradable materials in this work was driven by the need to find possible solutions to two major problems in the food sector that have an impact on both the environment, human health and the economy, namely high food waste and the huge volumes of non-biodegradable plastics food packaging wastes. To meet these needs, this work focused on the development of active packaging materials that were both biodegradable and active in maintaining the shelf-life of perishable foods. The active agents investigated were antimicrobial compounds, such as vanillin, antioxidants, such as spent coffee grounds extract (always with a view to the circular economy) and alpha-tocopherol, and the action of natural materials capable of acting as thermal buffers, such as oleic and linoleic acid was also investigated.

The techniques adopted for the production of polymeric materials have all proved to be suitable for the treatment of both biopolymers and known heat-sensitive active substances. In particular, it was possible to obtain completely different polymeric structures starting from the same polymer, which responded differently to the investigated requirements. On the one hand, the solvent casting technique made it possible to produce dense and transparent zein films with moderate release kinetics of the loaded active compounds over time, compared to the electrospinning technique, and with the possibility of modulating the release simply by varying the amount of active compound loaded. Furthermore, these materials showed good mechanical properties in terms of tensile strength, but were mediocre in terms of elongation at break and modulus of elasticity due to the brittleness typical of this biopolymer. The loaded compounds, and in particular the spent coffee grounds extract, did not affect these mechanical properties while they showed to improve the oxygen barrier capacity compared to the pure polymer, obtaining satisfactory results compared to what is reported in the literature. Given the results obtained for these zein films produced by casting, a possible application

to which they could be destined is as primary packaging, or as a layer in contact with food in multilayer packaging for food such as dried fruit and baked goods considering the vanillin flavoring or for foods with a hydrophilic character considering the good response obtained from the migration tests conducted in the food simulant ethanol at 10% v/v.

The electrospinning technique, on the other hand, allowed the fabrication of a more complex structure composed of micro and sub-micrometric fibers arranged in a spider web. This type of polymeric structure proved to be unbeatable in terms of release kinetics, as the large surface offered by the fibers allows for very rapid release kinetics, making this type of material suitable for fresh products to be consumed within a few hours, such as chopped fruit and fresh meat or fish. Given the opaque nature of the material, it is difficult to think of its use as primary packaging for fresh products that usually require transparent packaging, therefore a potential use could be to use them as antimicrobial or antioxidant patches to be inserted inside the packages for preventing the growth of bacteria, yeasts or molds. Furthermore, this use could also be a good solution considering the poor mechanical performance and the zero-barrier capacity to gases due to its porous nature. To improve these properties, however, various strategies are available including coupling with other polymers for making polymeric blends, making multilayer films, or surface treatments such as cold plasma to make the surface hydrophobic.

Another green process investigated for the treatment of these materials and in particular for the indirect loading of the active compounds was the impregnation process with supercritical fluids which allowed, in the case of zein, not only to load the compound successfully but also to obtain a purification of the polymeric structure from solvent residues and lipophilic impurities intrinsic to the zein. By means of this technique, release kinetics have been obtained, also in this case which can be modulated according to the quantity of active compound loaded.

In the case of polycaprolactone it was possible to optimize the operating conditions both of the sample production phase and of the alpha-tocopherol impregnation phase to obtain also in this case materials with excellent release kinetics suitable for use as packaging components for foods sensitive to lipid oxidation. Finally, as regards the production of polycaprolactone films enriched with phase change materials for the production of food packaging capable of counteracting the thermal fluctuations that food undergoes during the cold chain, the loading of these compounds inside the polycaprolactone fibers was successful, but the loaded quantities proved to be too smalls to be able to saturate the bonds with the polyester remaining also free to respond to changes in temperature. For this, it will be necessary to increase their content or encapsulate them inside particles to protect them from migration and in turn load the particles inside the packaging material. However, considering the almost non-existent literature concerning the direct loading of phase change materials of natural origin into a biodegradable polymer through a green technique, even these preliminary results can be considered a small milestone for what will surely become a topic of wide interest.

Finally, considering the potential development of electrospinning technology on an industrial scale for the production of active food packaging, which, as highlighted by the proposed business plan model, has proved to be profitable, it will be necessary to create a model that describes the process and that can predict the desired fiber size without having to conduct a feasibility and morphological optimization study each time the polymer or active substance is changed. In particular, fluid dynamics modeling relating to the formation of the Taylor cone through the application of the Navier-Stokes equations has already been extensively investigated in the literature. From what emerged during this Ph.D., it would be interesting to investigate how to make the process independent of the choice of polymer. In detail, it was seen how the electrospinning process is dependent on numerous parameters relative to the solution, such as polymer concentration and in particular the viscosity of the solution, as well as conductivity and operating parameters such as voltage and flow rate. It emerged that these parameters must be varied in a specific way as the materials vary in order to optimize the morphology of the product.

Therefore, the idea identified is to build a model not only capable of predicting the morphology as the input conditions vary, but on the contrary that is capable of providing indications on which parameters it is necessary to use to obtain a given morphology, especially considering the great variability of natural polymers, even if only using different production batches. To achieve this goal, the idea is to use black-box models and in particular, Artificial Neural Networks (ANNs), using the data collected in this work as a training database in order to be able, once the model is completed, to standardize the solution to be processed by identifying the optimal characteristics that a solution to be electrospun must have, and so that the program provides, depending on the input data supplied to the neural networks, indications on the possible operational changes to be implemented before processing the solution, such as for example, increasing the viscosity of the solution, increasing its conductivity by adding salts, etc.

In conclusion, all the processes adopted in this project allowed to work in mild conditions while preserving the nature of the processed compounds, and allowed to obtain films with different properties. For each material and for each technique used, the strengths and weaknesses were identified and interesting solutions were proposed to improve the properties required of food packaging materials. The materials developed in this work proved to be promising and may offer a valid alternative to traditional plastic packaging, with potential beneficial effects on food and the environment.

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